Central amygdala microcircuits mediating learning and expression of passive and active defensive behaviours

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Synopsis

The survival of all living beings is dependant on their ability to detect and adapt to environmental changes. Especially in the face of threat, the modus operandi needs to be adjusted in order to minimise potential harm and increase the likelihood of survival. Animals not only adapt their physiology, but also their behaviour. A large body of evidence shows that amygdala is a region of the brain crucially involved in innate and learned defensive behaviours. Its role in encoding passive defensive reactions elicited by classical fear conditioning has been thoroughly studied both on molecular and circuit levels. However, not much is known about amygdala circuits involved in active defense, such as flight.

Here, the role of central amygdala protein kinase C - delta ($PKC\delta^+$) and somatostatin (SOM^+) expressing neurons in both defensive freezing and flight was explored with the help of a two-way active avoidance conditioning paradigm. Optogenetic activation of $PKC\delta^+$ neurons resulted in boosted learning and expression of active avoidance. In contrast, activation of SOM^+ neurons had the opposite effect on behaviour. Additionally, calcium imaging of $PKC\delta^+$ neurons showed that a seizable fraction of this population changes its activity to conditioned stimuli in the course of active avoidance learning. Using optogenetics and imaging, we thus confirm that central amygdala mediates active defensive behaviours. Furthermore, we specifically identify that $PKC\delta^+$ neurons not only regulate passive, but also active defensive behaviours.

$PKC\delta^+$ neurons are thus in a position of power, which allows them to influence very different defensive strategies acutely and flexibly. Additionally, they also optimise adaptation to threatening situations in the long run via their lasting effects on learning.

Abbreviations

AAV	Adeno associated virus
ARCH	Archaeorhdopsin
BA	Basal amygdala
BLA	Basolateral amygdala
BNST	Bed nucleus of stria terminalis
LA	Lateral amygdala
CEA	Central amygdala
CEl	Central lateral amygdala
CEm	Central medial amygdala
ChR	Channelrhodopsin
CR	Conditioned reaction
CS	Conditioned stimulus
EFF	Escape from fear
HC	Hippocampal formation
IN	Interneuron
mPFC	Medial prefrontal cortex
PAG	Periaqueductal gray
PKCδ^{+}	neurons expressing protein kinase Cδ
PKCδ^{-}	neurons **not** expressing protein kinase Cδ
PN	Principal neuron
SSDR	Species specific defensive reaction
UR	Unconditioned reaction
US	Unconditioned stimulus

Introduction

1.1 Universality and diversity of defence systems: a snapshot

The survival of all living beings is dependant on their ability to detect and adapt to environmental changes. Especially in the face of threat, the modus operandi needs to be adjusted in order to minimise potential harm and increase the likelihood of survival. All organisms need to detect and correctly respond to physical changes of the environment, such as low nutrient conditions, oxidative stress, extreme pH and temperatures. For example, when exposed to high temperatures *Escherichia coli* bacteria induce transcriptional up-regulation of heat-shock proteins that in turn coordinate cellular repair mechanisms. Some heat-shock proteins help reverse protein misfolding caused by high temperatures and others tag terminally denatured proteins for degradation, thereby reducing concentrations of harmful misfolded proteins in the cell[Vabulas et al., 2010]. This is an example of metabolic adjustment to a physical change in the environment. Similar mechanisms exist in most organisms, including humans.
Multicellular organisms of the Plantae kingdom can also encounter environmental stressors such as extreme temperatures, excess light, draught, salinity, parasites and herbivores. Due to their very limited mobility, plants developed various physiological strategies of dealing with threats that can be classified as metabolic, active or passive mechanical and chemical shielding. For example, *Arabidopsis thaliana* employs an active mechanical strategy in order to protect itself from draught. Low-water conditions launch transport of abscisic acid to stomatal guard cells, where it triggers a signaling cascade that results in a reduced guard cell turgor and subsequent closure of stomata. Their closure reduces additional water loss in form of vapour via these structures, which primarily serve as CO_2 gas exchange routes.[Osakabe et al., 2014]

In addition to physical environmental stress, organisms face threats from other living

beings. Both conspecifics and heterospecifics can be sources of danger. *E.coli* can be attacked by phages, but also exploited by certain animals as a nutrient source. At the same time, instead of just passively consuming food when available, they also actively compete with other bacteria for favourable niches. To this purpose, they can produce defensive compounds like antibiotics, thereby weakening non-resistant bacteria in the proximity.[Chung et al., 2006; Hibbing et al., 2009]
Multicellular organisms also face attacks by heterospecifics, be it unicellular or multicellular, be it parasites or predators. In plants, the first line of defence against herbivores is mechanical - thorns, spikes and tychomes are common in many plants, including citrus trees and cacti. A chemical battery is often used as the second line of defence. For example, sharp oxalate crystals and various poisonous substances are packed in specialised cells called idioblasts of *Dieffenbachia* plants. Upon rupture of plant tissue, which occurs during attacks by herbivores, idioblasts burst and release these harmful substances that can cause painful irritation of animal mucus and skin. [Franceschi and Nakata, 2005]
Animals can also use chemichal and mechanical strategies in order to minimise the likelihood of being eaten. The hard shells of turtles, keratinous spines of hedgehogs and calcite spines of sea urchins are all examples of mechanical anti- predator systems. Some sea urchins, like those of the *Diadematidae* family, employ a combination of mechanical and chemical defence - they have hollow spines that are filled with toxins [Russell and Saunders, 2016; Tsafnat et al., 2012]. Many insects are capable of chemical defence, but they can also excel in camouflage. The walking stick insects resemble thin tree branches - a feature that allows them to blend in perfectly into their natural habitats, thereby reducing the probability of getting detected by predators [Hennemann et al., 2015]. Chameleons are capable of active camouflage, which allows them to flexibly adapt their skin colour and pattern to their environment [Stuart-Fox et al., 2008]. Another related strategy is mimicry. Instead of blending into the surroundings, some species are thought to have evolved to physiologically resemble another animal species. A famous example is the so-called Müllerian mimicry, a phenomenon that describes two unpalatable species resembling each other. Due to the resulting abundance, both profit from being more likely to be remembered as unpalatable by predators. [Meyer, 2006]

Animals do not rely on physiological defensive mechanisms only, but extend defence to the behavioural domain. Just like physiological adaptations, behavioural acts of self preservation can be observed in response to threatening physical environments or threatening biological interactions. The spectrum of these behaviours is characterised by a complexity and breadth at least matching that of physiological adaptations to

threats. It includes behaviours like aggregation, where animals form social groups that help increase success during foraging and reproduction, and promote survival. The latter is accomplished by confusing predators on one hand, and by increasing efficiency of predator detection by the group on the other.[Lima, 1995; Milinski and Heller, 1978] Nocturnality, which is the predominant circadian behaviour in rodents, is postulated to have evolved from a need of prey to minimise contact with diurnal predators and to reduce the amount of interspecific competition.[Gerkema et al., 2013] Predator satiation is another strategy employed by insects and some amphibians. Periodical cicadas emerge from their underground hide-outs every 13 or 17 years at extremely high densities of more than 300 individuals per square meter. For a short time they are thus easy prey for predators, however due to the extremely large numbers and short time of abundance, their predators are quickly satiated. This ensures survival of most individuals of the group.[Dybas and Davis, 1962] Phagomimicry is an example of employment of deterrent signals to distract and warn predators. Upon detecting danger, sea hare *Aplysia californica* releases a cloud of ink and a chemical coctail form the opaline gland containing high concentrations of taurine. The latter is known to be a powerful feeding signal in lobsters, which are sea hares' natural predators. Instead of trying to consume the aplysia, lobsters make feeding attempts for the chemical cloud, thereby giving aplysia a chance to escape. [Eisthen and Isaacs, 2005; Kicklighter et al., 2005] In deimatic behavior, animals signal false threat to predators or competitors, thus misleading them into flight. For example, fork-tailed drongos use deceptive false alarm calls to trick other birds engaged in feeding into flight thus involuntarily surrendering their food to the drongo.[Flower, 2011]

All these are examples of either highly specialised or rather complex defensive behaviours. There are however simple syllables of defensive behaviour, some of which are evolutionarily conserved, that are integral parts of more complex defensive strategies. Two examples of such syllables are immobility and flight. Flight is a behaviour in which animals try to minimise harm by introducing significant physical distance to the threat in a short amount of time. Immobility, or "freezing", is a behaviour in which an organism reduces locomotion to a halt, in an attempt to remain undetected by a predator. Both behaviours, although varying in details, can be found in species ranging from insects to humans. Their simplicity and evolutionary conservation makes each of them a good model system for studies of defensive behaviours.

1.2 Defensive behaviour in humans

As is true with other animals, the survival of humans crucially depends on their ability to adapt behaviour in the face of threat. Humans have an innate threat- defence system that can be extended and modified by learning. Stimuli like loud noises, unexpected and sudden movements, as well as pain-eliciting events like touching a sharp needle or a hot plate are instinctively recognised as harmful. Such stimuli are also known as unconditioned stimuli (US). The aversive US listed here are capable of reliably provoking innate defensive reactions, also known as unconditioned reactions (UR). For completeness sake, it is worth noting that unconditioned stimuli are not always aversive and can have positive or negative valence, or even be perceived as ambivalent. A positive valence US can for example provoke appetitive or consummatory behaviours. On contrary, an aversive US will provoke defensive UR under normal circumstances. [Myers et al., 2014]

Touching a hot object is an event that triggers a defensive UR - a spinal cord coordinated nociceptive withdrawal reflex. This reflex has protective qualities, as it introduces distance between the subject experiencing the noxious stimulus and the hot object. [Campbell et al., 1991; Willer et al., 1979] In case of aversively loud noise, another set of reflexes is triggered and coordinated by the brain stem. It consists of a combination of several stereotyped action patterns that include a jaw reflex, eye blink reflex, and jerks of shoulders and legs. This action pattern serves to protect the eyes, head and neck, and to prepare for escape. [Grillon et al., 1997; Landis and Hunt, 1939] Apart from reflexive reactions, more complex and adaptable URs like immobility, escape and defensive aggression are also part of the human defensive repertoire. It appears that the relationship between threat, environment and such behavioural outcomes is not deterministic, but probabilistic. Thus, the categorisation of immobility, flight and fight as reflexive is not justified, in spite of their simplicity. Rather, they are simple defensive behaviours with an instinctive basis, that are induced upon extensive assessment of the environment.

More complex defensive reactions that are acquired by (instrumental) learning exist as well. These cannot always be classified as defensive unconditioned reactions, as they do not necessarily occur naturally in the context of a threat, but do so only upon conditioning. In fact, any action that reduces perceived or actual threat levels has a strong potential of being reinforced and becoming an instrumental avoidance behaviour. [Cardi et al., 2015; Collins et al., 2014; De Silva and Rachman, 1984; Mowrer, 1939].

Defensive reactions in humans can be accompanied, modulated and even triggered by emotions. There are many definitions of emotions in the scientific literature. Although a consensus has yet to be reached, one appealing definition is that they are reactions to stimuli that engage the entire organism, and are simultaneously composed of physiological, behavioural and cognitive components. Two emotions with tight links to defensive reactions are fear and anxiety. [Myers et al., 2014]

1.3 Fear and Anxiety

Similar to the chemical defence battery in Aplysia described above, fear and anxiety have developed in the course of evolution in order to improve chances of survival in the face of threat. Both emotions are a part of the human threat defence system and both are negative affects that are hallmarked by elevated vigilance, uneasiness and tense apprehension. Fear is an acute, intense emotional reaction in response to a well- defined and perceived threat. It is induced rapidly upon detection a threat stimulus, and it dissolves soon after danger fades away. Anxiety on the other hand can last longer, its beginning and end are diffuse, as are the stimuli inducing it. It is usually initiated by an anticipated, not perceived danger. Generally, anxiety is less intense than acute fear, but its persistence makes it, for the most part, very unpleasant to people experiencing it. Although unpleasant, fear and anxiety are adaptive emotions when triggered in response to perceived or potential danger. They have the capacity of orchestrating immediate defensive reactions, as well as sustainably adapting behaviour in a manner that improves coping with similar stressors in the future.[Davis et al., 2010; Myers et al., 2014; Rachman, 2013]

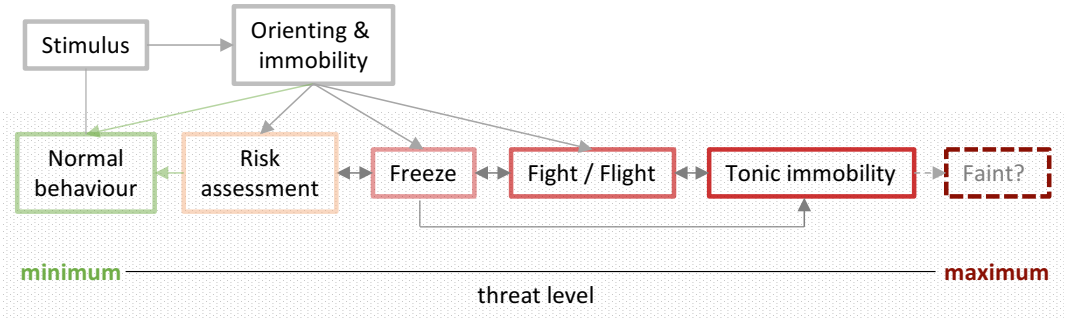

Figure 1.1: Simplified model of human human defensive behaviours. Adapted from Hagenaars et al. [2014]

In line with the definition of emotions, and justifying their classification as such, adaptive fear and anxiety induce not only a change of affective state, but are also accompanied by physiological and behavioural changes. In fact, even detection of a sensory stimulus, irrespective of its salience, is usually followed by an inhibition of ongoing behaviours and a subsequent induction of attentional processes. The latter is characterised by orienting behaviour if the stimulus is novel, and by attentive immobility.[Hagenaars et al., 2014; McNaughton, 1993; Steimer, 2002] This type of immobility is accompanied by parasympathetic activation, including bradycardia and simultaneous mobilisation of attentional resources. [Hagenaars et al., 2014] This phase has a short duration and latency, however significant differences in processing time have been found for salient and non-salient stimuli. In cases where the detected stimulus poses a potential threat, risk assessment behaviours associated with an anxiety state are induced. These include scanning the environment, tendency to lean towards the stimulus and even approach it. If threat levels are categorised as low, the human will go back to normal behaviour and so will the autonomic system.[Blanchard et al., 2001b, 2011; Hagenaars et al., 2014] If the situation is classified as dangerous, the fear pathway is induced. In cases in which escape in not possible, humans are sometimes reported to start freezing. This phenomenon was first observed in animals [Blanchard et al., 1968], however humans seem to show related changes in posture in response to intermediate threat levels [Hillman et al., 2004; Horslen and Carpenter, 2011; Mouras et al., 2015; Stins and Beek, 2007]. This kind of immobility has evolved to reduce probability of being detected by predators and is preferred in situations where escape is not possible. This state is accompanied by both sympathetic (pupil dilation) and parasympathetic (bradycardia) activation. This is thought to further increase attention, sensory perception and threat assessment. As the time progresses, sympathetic activation becomes dominant and results in metabolic activation that prepares for defensive action. [Bradley et al., 2001]

It is worth noting that many times when "freezing" is reported in studies of human behaviour, it refers to attentive immobility. Some authors however argue that these are two separate processes. The differences are subtle: intensity of bradycardia is somewhat higher in fearful freezing, and the immobility is longer in duration and intensity. Another interesting observation is that during fearful freezing, one can observe startle potentiation, whereas this does not occur during attentive immobility. [Vrana et al., 1988] This has been used by Bradley et al to distinguish between attentional processes that dominate attentive immobility, and the fearful state that dominates during freezing. [Bradley et al., 2001]

At high threat levels in environments where escape is possible, rapid sympathetic activation increases further and culminates in flight. If escape is not possible, the same threat is likely to induce defensive aggression. [Blanchard et al., 2001b; Davis et al., 2010; Harrison et al., 2015; Steimer, 2002] This is the famous fight-flight reaction which was first described by Cannon in the early 20th century [Cannon, 1975]. From the ethical perspective it is difficult to justify exposure of human subjects to threat levels so high that they can induce fight or flight responses in experimental settings. This is why most laboratory studies focus on hypothetical threat and fight/flight responses. Anecdotal evidence of such behaviours exists from mass panic incidents during natural disasters, ship collisions, on soccer stadiums, concerts and large religious festivities [Elliott and Smith, 2016; Fritz and Marks, 1954; Illiyas et al., 2013]. These suggest that flight is likely to be induced in situations where an escape route is present, but the window of opportunity for escape is limited and diminishing. The rise of video surveillance, as well as recent attempts to model flight behaviour in computer simulations might yield further insights. [Helbing et al., 2000; Moussaïd et al., 2016]

Tonic immobility, also referred to as apparent death or thanatosis, is considered to be the last resort of human defence and is induced only in extremely life-threatening situations in which an escape is not possible. This state of profound immobility is an attentive, alert state that is hallmarked by intense activation of the sympathetic nervous system. This includes tachycardia, which is also a feature that sets it apart from "simple" freezing. [Alves et al., 2014; Volchan et al., 2011] Interestingly, tonic immobility often ends suddenly with either flight or defensive aggression. [Abrams et al., 2009; Heidt et al., 2005; Volchan et al., 2011]. It is worth noting that some authors have recently argued that "faintness", the propensity of a small percentage of the human populations to loose consciousness upon seeing blood, wounds or needles, should be added to the list of basic syllables of defensive behaviour [Bracha, 2004; Bracha et al., 2007; Kleinknecht, 1987; Olatunji et al., 2006].

Other defensive behaviours like hiding, defensive threat, alarm vocalisation, tend and befriend also exist, but are not quite as well studied. Their defensive direction and relationship to defensive intensity are current focus of some research. [Blanchard et al., 2001b; Krupić et al., 2016; Perkins and Corr, 2006]

As mentioned previously, fear and anxiety are adaptive human emotions. However, if they are repeatedly triggered in absence of potentially threatening stimuli, if they persist way beyond the cessation of danger, or if their intensity is disproportionate

to the magnitude of actual threat, then fear and anxiety can become debilitating and maladaptive. The prevalence of anxiety and panic disorders in the western world is striking. Studies estimate that 10 to over 30 percent of humans suffer from anxiety disorders at some point during their life. [Bandelow and Michaelis, 2015; Kessler et al., 2005; Steel et al., 2014] Although highly variable, the undoubtedly high prevalence, combined with their negative effects on life quality and high treatment and economic cost [Greenberg et al., 1999; Kessler and Greenberg, 2002], make anxiety disorders important objects of research in modern psychology and neuroscience.

1.4 Rodent models for studying human emotions

Current research on the nature and biological basis of fear and anxiety in humans is limited by several factors. On one hand, exploring the full emotional repertoire in the laboratory is limited by high intersubject variability, monetary and time constrains. Since fear and anxiety involve negative affect, ethical concerns are not uncommon. Studying the biological basis of emotions calls for exact multi-parameter recordings of human behaviour, physiology and brain function. Here, great care is given to employ minimally invasive technologies. However, such state of the art technologies often suffer from suboptimal data sampling and from being invasive to the behaviour. One such example are the functional magnetic resonance imaging (fMRI) devices. The bulky instruments require the study subjects to lay still for the duration of the experiment, thus limiting possibilities for interaction with the subject and limiting their behavioural repertoire. The spatial and temporal resolution of fMRI has improved considerably, yet it is still far from giving precise accounts on firing patterns of single neurons. Additionally, fMRI currently measures indirect reports of neuronal function based on blood flow. Other methods, like electrophysiological recordings can directly measure neuronal firing in a fast and direct manner, but they suffer from being highly invasive (implantation of electrodes) and small sampling volumes. The sheer size and complexity of the human brain in itself poses an immense challenge. Observation of complex systems without interfering with their function makes statements about causality of observed phenomena difficult and risky. Interference with the behaviour can and is successfully applied in psychological research. However, invasive manipulations of neuronal function are an ethical concern in healthy human subjects, especially when they involve novel viral, optogenetic and pharmacogenetic techniques. These do have great potential for precise manipulation of neuronal firing necessary for delinating the contriubtion of neuronal circuits to

emotions, but they also leave a potentially lasting and not well characterised footprint in the brains of treated subjects.

Although not as well studied from the evolutionary standpoint as morphology and cell biology of animals, behaviour has already been explored from the phylogenetic perspective in the late 19th century by Darwin. [Darwin, 1872] However, the study of emotions in animals has some limitations that are intrinsic to how we define emotions. As mentioned previously, psychologists view emotions as states that involve the entire organism and that are hallmarked by changes in physiology, behaviour and cognition. The accurate assessment of the latter is currently thought to be possible only by verbal account, which is impossible in animals. It is thus unclear weather animals have emotions and if so how they compare to human experiences. Nevertheless, the study of behaviour and the accompanying physiological changes is very well possible and meaningful. Rodents exhibit defensive behaviours that resemble those observed in humans. Examples are the acoustic startle reflex, risk assessment, freezing, escape and defensive fight. Additionally, these behaviours are accompanied by altered physiology that includes changes in blood hormonal levels, heart rate, blood pressure, muscle tone and pupil size. The fact that in the face of threat both behaviour and physiology in rodents co-vary in a manner similar to that observed while humans are experiencing fear and anxiety is a hint that such full-body reactions in rodents can be a viewed as a reduced model of these human emotions. Additionally, the anatomy of the central nervous system in humans and rodents bear some similarity. Neuroanatomical units grossly homologous to human thalamus, hypothalamus, cortex, amygdala, cerebellum and brain stem are found in rodents. Also, some fine scale anatomical features like the proximity of serotonergic cells to the fourth ventricles are found in both rodents and human. There are of course very obvious differences between human and rodent nervous systems. The much smaller size and complexity of the rodent brain is not necessarily a drawback. In fact, this is one of the feature that make the neuronal circuits more accessible for investigation with currently available techniques. Other favourable features are the genetic and viral accessibility of mouse neurons. These allow ever finer and more precise control.
Ethical concerns about the use of rodents as experimental animals in neuroscience are valid and need to be addressed. The scientific community is obliged to respect the life of laboratory animals and to minimise their suffering and pain during experimental procedures. Reduction of numbers of laboratory animals to the minimum needed to reliably answer a scientific question, replacement of animal experimentations with alternative approaches wherever meaningful, and constant refinement of the experimental methods

are of great importance for meaningful and humane research. At the moment, we know still so very little about the biological basis of normal and pathological behaviour and emotions that we cannot rely exclusively on models that do not involve a study of a living being. Without the latter, the research community can not make continuous significant progress in understanding fear and anxiety. However, we can lay out a path for a future where this might be possible, at least in part by, in parallel to performing animal research, developing mathematical concepts needed to build theoretical constructs of brain function.

In summary, although brain anatomy, defensive behaviour and physiology show obvious differences between humans and rodents, they also show many similarities that justify using the rodent defence system as a model for the biological basis of human emotions of fear and anxiety.

1.5 Defensive behaviours in rodents

Although, the details of defensive behaviours are species-specific, the general defining features seem conserved in mammals, including humans. The most prominent behaviours displayed in threatening situations in mice and rats are risk assessment, freezing, flight, fight, hiding, defensive threat, and defensive fight. [Blanchard et al., 2001a] This largely corresponds to the unconditioned defensive behaviours in humans. Unconditoned stimuli that can elicit defensive behaviours are also somewhat conserved. For example, like in humans, sudden loud noises induce the startle reflex in mice and rats. This reflex is characterised by short latency (less than 10ms) activation of skeletal muscles culminating in a jerk. The jerk itself involves the entire body - rodents first extend their paws and then flex into a crouch.[Davis, 1980; Parham and Willott, 1988] Distant threats or diffuse stimuli like brightly lit, open spaces and heights provoke risk assessment behaviours. These include avoidance, freezing, rearing, stretch-attend postures and grooming. The co-occurence of these behaviours with heightened defecation and urination are indications that a simultaneous activation of the sympathetic nervous system occurs.[Bourin and Hascoët, 2003; Hascoët and Bourin, 2009; Rodgers and Johnson, 1995; Simon et al., 1994; Treit et al., 1993] Proximal, well defined threats distinctively induce freezing, hiding or flight. Some of the threatening natural US are species specific - for example, prominent mouse predators are cats, foxes and predatory birds like owls and hawks. In the course of evolution, mice have developed sensitive systems for extracting cues about presence of exactly these predators from the environment. For example, odour of the fox

urine can elicit defensive URs in mice and rats. As predator odours are, like any natural odour, somewhat diffuse in time and space, they induce risk assessment behaviours in addition to freezing, avoidance and potentiation of startle responses. [Apfelbach et al., 2005; Hebb et al., 2003; Wallace and Rosen, 2000] Trimethylthiazoline, a chemical compound found in fox urine, elicits anxiety-like behaviours in mice that are accompanied by sympathetic activation hallmarked by increased defecation, accelerated heart rate, serum corticosterone and analgesia [Fendt et al., 2005; Yang et al., 2016] Visual stimuli that are in accord with an attack by a predator form the air, for example looming visual cues, are better predictors of threat imminence than olfactory cues. Instead of evoking anxiety-like behaviours, they act as strong US that evoke short latency fear-like flight or freezing. [De Franceschi et al., 2016; Ellard and Goodale, 1988; Shang et al., 2015; Wei et al., 2015; Yilmaz and Meister, 2013] Painful stimuli that have the potential of causing tissue damage induce escape, jumps, alarm vocalisations and freezing.

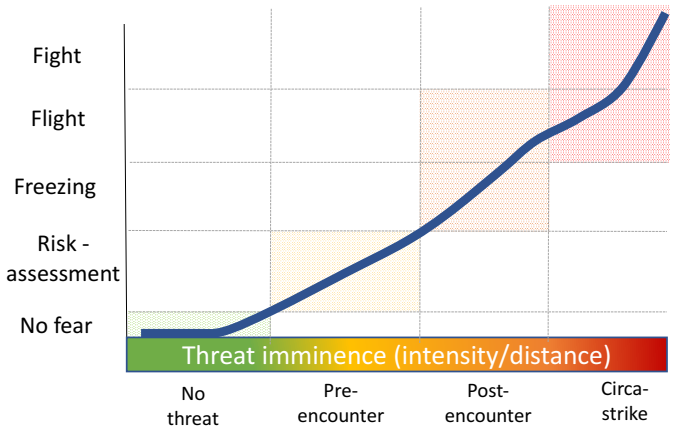

Figure 1.2: Simplified model of the organisation of rodent defence behaviour according to the threat imminence theory. Adapted from Blanchard and Blanchard [1988]; Fanselow and Lester [1988]

In summary, the more diffuse or distant a threat stimulus, the more likely it is to elicit risk assessment behaviours. Proximal and well defined threats are more likely to provoke freezing, flight or fight. This rule of thumb has been formulated more precisely in the theory of threat imminence, which was originally named predatory imminence theory. [Fanselow and Lester, 1988] An encounter with a predator or threat is divided into three stages that depend on the perceived threat distance. In the pre-encounter stage, the threat has not been unambiguously determined, but the presence of clues in the environment points in the direction of a potential threat. In this stage, the animal performs behaviours that help minimise the chances of an encounter with the preda-

tor. The animals increase their vigilance, avoid brightly lit and open spaces, and move with a cautious, stretched posture. During the somewhat ambiguously named "post - encounter" phase, the animal has clearly detected a threat and employs behaviours that could reduce the detection or attack by the predator. Freezing is a typical post-encounter defence, during which the animal stops all movement, except for breathing, for at least a few seconds. This is a highly vigilant state in which the animal's body and muscles are tense and prepared for a flight or fight response. During the "circa-strike" stage, the predator has detected the animal and is attacking it. Under such circumstances, mice react by vocalising and making escape attempts when an escape route is available, otherwise they perform a series of defensive attacks. Studies from the Blanchards' and others suggest that the intensity of threat also plays a critical role during the selection of a defensive strategy [Blanchard and Blanchard, 1988; Blanchard et al., 2001a; Fanselow and Lester, 1988; Perusini and Fanselow, 2015]

In addition to a classification of defensive behaviours according to the predatory imminence spectrum, these behaviours can also be categorised as "active" or "passive". Active defence includes flight, jumps and defensive aggression. Sometimes, risk assessment behaviours are also counted as active. Passive defence is hallmarked by freezing and tonic immobility.[Bandler et al., 2000b; Gozzi et al., 2010] It is currently debated wether the later is a natural behaviour in mice, although there are indications that mice can enter a thanatosis-like state [Bazovkina et al., 2011; Kulikov et al., 1993; Webster et al., 1981] These two categories are not to be confused with (pro-)active and passive coping in humans. Coping is a conscious cognitive effort to deal with a stressful or threatening situation. Since coping is limited to conscious cognitions by definition, it does not include unconditioned defensive behaviours. [Brown and Nicassio, 1987; Carver and Connor-Smith, 2009; Snow-Turek et al., 1996]

1.6 Learning about threats

The power of the innate defence system is its ability to enable animals to respond quickly and effectively with appropriate counter-measures to evolutionarily established threats in a situation tailored manner. Apart from the described effects on behaviour and physiology of animals, activation of the defence system also promotes memory formation. [LeDoux, 2012] In other words, stimuli that can elicit unconditioned defensive reactions are the ones that can act as reinforcers in learning. A likely purpose of the effect on memory formation is its potential to optimise survival in dangerous situations based on

previous experience. This could for example be achieved by enhanced sensory detection and discrimination of threat from harmless stimuli. Other possible optimisations are more streamlined action selection, and more threat and environment optimised performance of the selected defensive action.

The search for specific features of threats that can promote memory formation is an ongoing effort. There are strong indications that pain is one of those features, and yet it is disputed what its evolutionary value as a reinforcer could be. Some authors argue that once it comes to a predatory attack (and thus pain), survival is unlikely and hence learning opportunities and evolutionary advantages very limited. [Bolles, 1970; Bolles and Fanselow, 1980; Cushman and Fanselow, 2010]. According to this line of argumentation, more advantageous learning is the one that occurs upon threat detection, for example associative learning that connects environmental cues to the detection of threat, instead of connecting them to the attack. The idea behind this is that cues that predict the occurrence of a threat provide animals with an opportunity to avoid an attack before it happens. Previously named natural US like predator odours, looming visual stimuli and loud noises are features that can be detected before an attack. An example of a pain-inducing, but well controllable, artificial US in laboratory conditions is electric foot shock. It is still unclear how foot shocks compare to natural US and weather they might resemble pain that prey experiences during an attack. There are hints that although an electric shock results in similar behaviours as observed during an encounter with a predator or a hostile conspecific, the brain regions engaged are at least partially different. [Silva et al., 2013]

Memory promoting effects of an activated defence system are exploited in the laboratory to study neuronal basis of learning and threat processing. In the so called classical fear conditioning (or threat conditioning), an alteration of Pavlov's original associative learning paradigm, an originally neutral stimulus that does not naturally have the power to bias behaviour towards defensive actions, commonly referred to as a conditioned stimulus (CS), is paired with an aversive unconditioned stimulus. [Ledoux, 2014] Tones are commonly used CS, since they are well controllable in intensity, amplitude, and have a fairly predictable spatial spread. In most studies, they are paired with mild electric foot shocks (US), although tail shocks and eyelid shocks are not uncommon. Upon learning, which can occur after a single CS-US pairing, the CS comes to elicit an adaptive defensive behaviour, in this context referred to as a conditioned reaction (CR). The CR can resemble the UR, but does not always do so. Either way, the CRs are related to the eliciting US - for example, just like an aversive US will normally not come to elicit appetitive UR, it will also not elicit appetitive, but defensive CRs. The most prominent

CR elicited after successful tone-shock pairing in laboratory setting in which an escape route is not possible is freezing[Blanchard et al., 1968; Bolles and Fanselow, 1980]. Furthermore, upon conditioning the CS undergoes a "hedonic shift" - it adopts some of the features of the US and becomes aversive by itself. This not only enables it to elicit a defensive reaction, but it becomes empowered to drive associative memory formation in absence of a natural US. [Rescorla, 1973; Rescorla and Wagner, 1972] Apart from promoting memory formation and defensive behaviour, it also induces activation of the endocrine system and the sympathetic nervous system. CS are known to increase the level of glucocorticoids in the blood stream, tachycardia, blood pressure changes and potentiation of the startle reflex.[Brown et al., 1951; Liu et al., 2013; Tovote et al., 2005a,b] Noteworthy is that humans are also capable of this type of classical fear conditioning. [Eippert et al., 2012; Fullana et al., 2016] In fact, fear conditioning was first discovered in humans by John Watson. In a famous experiment with a young toddler Albert, an originally neutral stimulus, a white rat, was presented together with loud white noise as a US. After the CS-US pairing, a subsequent presentation of a white rat (CS) alone came to elicit distress in little Albert.[Ledoux, 2014]
As the activation of the defence system has negative aspects like higher energy expenditure and the cesation of ongoing behaviours like foraging, search for mates and nest sites, it is important that it is activated only when really needed. [Fernández-Juricic and Rodriguez-Prieto, 2010] This is why learning to shut down defence when unnecessary, is another important aspect of adapting to threats. A simple form of non-associative learning is habituation, in which behavioural responses to a (unconditioned) stimulus decrease in intensity with repeated exposures to that stimulus. Importantly, this process is not mediated by sensory adaptation[Rankin et al., 2009] An example for habituation learning is the attenuation of the startle reflex, which occurs upon repeated presentation of the loud auditory stimulus in both rodents and humans [Koch, 1999; Pilz et al., 2014; Wilkins et al., 1986] Interestingly, the inability of the startle reflex to habituate is observed in human patients with panic disorder and schizophrenia spectrum disorders. [Cadenhead et al., 1993; Ludewig et al., 2005; Takahashi et al., 2008] Conditioned defensive responding can also be decreased, for example by using a procedure called extinction. In fear extinction procedures, the fear conditioned subjects are repeatedly exposed to a CS without US pairing. This unreinforced exposure leads to a gradual decrease in the amount and intensity of displayed CR. [Myers and Davis, 2007] It has been shown that this phenomenon cannot be fully accounting for by the process of forgetting of the conditioned fear and that it includes new learning. [Baker and Azorlosa, 1996; Myers and Davis, 2007] Humans subjects that have been fear conditioned can also

reduce their fears via extinction. [Knight et al., 2004; Milad et al., 2007; Schiller and Delgado, 2010]
The most prominent CR elicited in rodents after successful classical fear conditioning in commonly used small plain contexts is freezing. More active forms of defensive behaviours like risk assessment and escape attempts are sometimes observed as well. Interestingly, female rats seem to have a natural preference for more active types of defence following classical fear conditioning [Gruene et al., 2015]. Additionally, rodents enhance active defence if provided with an escape route or a hiding spot. [Tovote et al., 2015] There are paradigms that have been composed in such a way that they preferentially elicit active defence. Examples of such conditioning paradigms are conditioned flight [Fadok et al., 2017], signaled two-way active avoidance [Mowrer and Lamoreaux, 1946], Sidman avoidance [Sidman, 1953] and platform-mediated avoidance [Bravo-Rivera et al., 2014], to name a few.

1.7 Avoidance learning

Avoidance is an umbrella term that is used to described certain types of learned defensive behaviours. It encompasses a complex set of learned behaviours that are loosely connected only by their ultimate purpose, namely the minimisation of threat. This is a fitting definition on one hand, but also a very unfortunate one on the other, since in the broad sense, most adaptive defensive behaviours, whether learned or not, are performed with the purpose of minimising threat. In rodents, avoidance can include both passive and active types of defence, for example escape, hiding, burying and even freezing or withholding certain behaviours. [LeDoux et al., 2017] Adaptive avoidance is an important survival strategy for humans as well. For example, avoiding contact with a sick person, or taking disease-preventing precautionary hygienic measures can help limit the spread of infectious diseases. In human psychology, the term avoidance is also used to describe a coping style (avoidance coping), characterised by behavioural or cognitive methods employed in order to avoid having to deal with the threat. It is usually an attempt to escape feelings of distress and it does not necessarily lead to a reduction in threat and distress levels. [1] If the avoidance coping is excessive, or even counter-

[1] "Coping" is a term from human psychology that describes conscious endeavours for dealing with threats. Dozens of different coping styles have been reported [Compas et al., 2001; Skinner et al., 2003]. Elimination of redundant terminology, proper characterisation and classification of coping strategies is currently incomplete and is one of the challenges of modern psychology. One of the more confusing instances about avoidance behaviour is that some authors associate it with passive coping styles, others

productive, it can become maladaptive. In fact, maladaptive avoidance is a hallmark of several psychological disorders, including generalised anxiety, panic and avoidant personality disorder [Carver, 2007; Rachman, 2013]

Avoidance is also used to describe a certain type of conditioning paradigms, in which the experimenter can set a criterion, that describes a behaviour which will lead to termination/minimisation of threats. Generally, avoidance conditioning paradigms have been classified as passive (also called inhibitory) or active. Passive avoidance conditioning includes all paradigms in which the animal has to withhold a certain action in order to avoid a US. Active avoidance conditioning on the other hand requires the animal to perform a certain action in order to avoid the threat.[LeDoux et al., 2017]

Active avoidance conditioning was first explored in the 1930s [Brogden et al., 1938; Dunlap et al., 1931; Hunter and Pennington, 1939; Hunter, 1935; Mowrer, 1940]. Dunlap and colleagues first described a two compartment apparatus with electric shock grid, connected by a walkway.

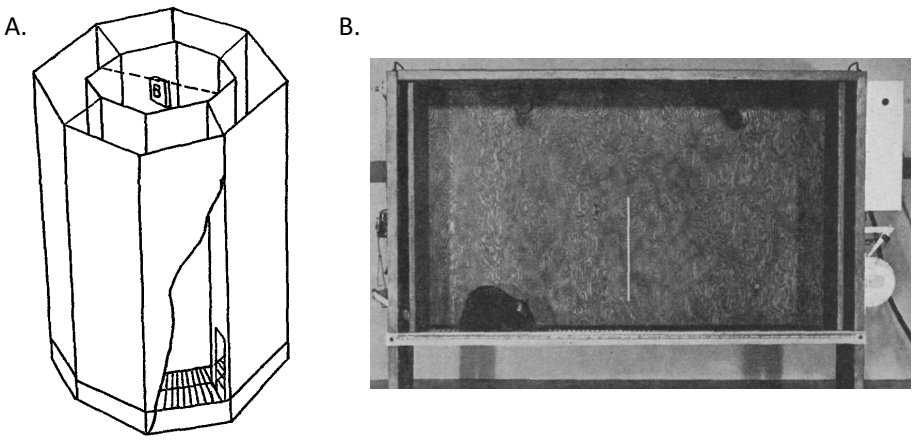

Figure 1.3: Two early designs of active avoidance conditioning setups. A. from Hunter [1935] B. from Mowrer and Lamoreaux [1942]

Upon application of shock, the experimental animals, rats in this case, had to learn that in order to terminate the application of shock, they had to cross to the other compartment via the walkway. Over the course of the experiment, rats started making targeted movements to the other compartment upon the onset of the shock, which the authors interpreted as shock escape-learning. Furthermore, they mention that after successful learning, the shock can be substituted by other stimuli - for example by tones.

with active. This topic clearly needs further discussion and research. Depictions of rodent active avoidance as a model for human active coping and Pavlovian threat learning as a model for passive coping are thus to be consumed with caution.

It is unclear if the tones in this study were at any point predictive of (or paired with) the shock, or if they were simply replaced by it, and unfortunately they give only descriptive account of rat's behaviour. [Dunlap et al., 1931]
In 1935, Hunter and colleagues published another early conditioning apparatus for active avoidance, in which eight sections with separate electric grid floors were connected with a walkway. A tone (buzzer) was turned on at certain intervals two seconds before the onset of the shock. Under these conditions, the tone is used as a CS and has predictive value of the US. If the rat transferred from one section to any other, the shock was not applied. [Hunter, 1935] In 1939 Hunter designed yet another active avoidance paradigm in which the experimental animals had to jump on or off a platform in order to avoid the application of a shock preceded by a tone [Hunter and Pennington, 1939]. It was however the first, circular design that at first gained most popularity. Using this paradigm, Brogden identified that the transfer of the escape response from the shock to the tone occurs only if this action prevents the occurrence of the shock. Inescapable shocks on the other hand caused not escape, but another reaction in the experimental animals (guinea pigs) "... when the tone began, they literally 'sat tight', held the breath, and tensely awaited the shock". [Brogden et al., 1938] To my knowledge, this is one of the earliest descriptions of conditioned freezing behaviour in rodents. In the 1940s, Mowrer performed several studies using different active avoidance paradigms. In one of the more interesting studies he used a modified version of Dunlap's setup and Brogden's auditory signalled avoidance conditioning. Placed in a square context separated in two halves by an imaginary line running through the middle, the experimental rats had to learn to avoid the US (shock) by making a targeted movement from one half of the context to the other during the CS (tone). This kind of conditioning paradigm is nowadays referred to as auditory signalled two way active avoidance. "Two-way" is descriptive of the fact that no matter which half of the experimental context the animal is in at the time of the CS occurrence, it has to transfer to the respectively other half. Interestingly, Mowrer found that the fastest acquisition of active avoidance in a signalled two way active avoidance task occurs when the CS (buzzer) precedes the application of the shock and co-terminates with it if no CR occurs, and when it terminates without subsequent shock as soon as the animal performs a CR.[Mowrer and Lamoreaux, 1942]

The most commonly used active avoidance task nowadays is auditory signalled two-way active avoidance. In this paradigm, the experimental animals are placed in a rectangular context with two nearly identical chambers. The floor of each chamber is lined with an electric shock grid and the chambers are split with a divider or a gate. The CS is a

tone of a few seconds duration, which predicts and is paired with a mild electric foot-shock. The shock and the tone are terminated instantly if the animal crosses through the gate during the CS-US presentation. Additionally, the shock can be avoided by shuttling through the gate during the tone-only period. Thus, upon successfully performing active avoidance, the tone is terminated immediately and the shock is not applied in that trial. Both mice and rats can learn to perform this task fairly well [Bignami et al., 1985; Choi et al., 2010; Darvas et al., 2011; LeDoux et al., 2017; Mowrer and Lamoreaux, 1942]. In the past, there have been several attempts to explain how and why the avoidance behaviour is learned. According to the two-factor (or "two-process") theory of learning, in the first stage of learning, a link between a CS and US is established via a Pavlovian associative learning. Because of the resulting connection of the aversive US with the CS, the CS itself becomes aversive (and capable of causing a "fearful" state). Then in the second stage of learning, a fear-avoidance link is established via an instrumental learning process.[Mowrer, 1951] How the later is established has been heatedly debated among researchers - the reinforcer [2] is not easily tangible. Two popular versions of the two-factor theory are the two-process fear mediation theory and the two-process aversion theory. [Masterson and Crawford, 1982] In the two-process fear mediation theory, the performance of the escape behaviour is thought to reduce the conditioned fear and this reduction is thought to be the reinforcer.[Mowrer, 1951] A related theory assumes that it is not the reduction of fear/threat that acts as a reinforcer, but the resulting safety state. Termination of CS following successful aviodance might act as a signal that predicts the absence of shock or a reduction of fear - both of which could be interpreted as "safety cues" that could foster further avoidance.[Bolles, 1970; Krypotos et al., 2015] In the two-process aversion theory, the "fear reduction" idea is discarded in the favour of a negative reinforcer. The CS which becomes aversive via the first, Pavlovian learning process, motivates the animals to remove it. Thus according to this model, the cessation of CS is the negative reinforcer that fuels avoidance behaviour.[Dinsmoor, 1954] The two-process fear mediation theory was criticised after experimental evidence showed that the active avoidance responses can be resilient to extinction. The reasoning was that each successful avoidance trial is in fact an extinction trial in which the CS is not followed by a US. In this case, a fear reduction and subsequent reduction of avoidant responding would be expected, but the latter was not observed. Another criticism that concerns both two-factor fear mediation and aversion theories fell on the claim that avoidance is acquired through an instrumental learning process.[Bolles, 1971; Krypotos et al., 2015]

[2] Stimulus that strengthens behaviour is referred to as a **reinforcer**. A **negative reinforcer** is a stimulus whose cessation strengthens a behaviour.[LeDoux et al., 2017]

Although this line of argumentation has had a heavy impact on the study of avoidance in general, it is in fact not based on evidence coming from the two-way active avoidance task. The claim that avoidance responding is not necessarily instrumental is based on an observation that some behaviours are more readily performed in various avoidance tasks than others. For example, jumping out of a box where conditioning took place could be learned in a single trial[Maatsch, 1959], whereas lever-pressing could not be efficiently acquired as an avoidance response[D'Amato and Fazzaro, 1966; DAmato and Schiff, 1964]. This motivated Bolles to argue that when under threat, the behavioural repertoire becomes limited to the species-specific defensive reactions (SSDR). Since only these behaviours were displayed, only SSDR and related behaviours could be utilised in an instrumental learning task. Jumping is one of those behaviours, as is running/flight. Lever pressing is not a SSDR and does not have the highest priority in threatening situation, which is in line with the observed difficulty with which it can be utilised as an instrumental avoidance response in rodents. [Bolles, 1970]. However, Bolles went even further and played with the thought that intrumental learning is not necessary in those circuimstances in which the learning can occur within a single trial. Instead, the observed avoidance might in fact be flight, a SSDR evoked in this case by the aversive properties (acquired during a purely Pavolvian learning process) of the CS[Bolles, 1971; LeDoux et al., 2017; Maatsch, 1959]

Current consensus is that further research is necessary in order to clarify weather avoidance is generally instrumental, but that it is also highly likely that two-way active avoidance involves an instrumental learning process. Furthermore, it is likely that there are several different reinforcers that contribute to active avoidnace learning, for example the CS-escape, US-escape, and the US-omission. Early evidence for the contribution of US-escape to active avoidance learning came already in the 1930s from experiments with guinea pigs in which the subject had to turn the cage rather than shuttle. In these experiments, inescapable shocks led to only a very low avoidance, whereas exapable shocks resulted in examplary learning after a week of training. [Brogden et al., 1938] First indications for (negative) reinforcing power of CS-escape were delivered by Mowrer and later by Kamin who observed that lower avoidance rates are observed when CS is not terminated at the time of shuttling in a 2wAA task.[Kamin, 1956; Mowrer and Lamoreaux, 1942] Although not perfectly clean, Kamin's study also shaped the ideas that US-omission contributes to optimal performance in a 2wAA task. Further evidence came from the "escape from fear" (EFF) paradigm, in which the CS-US contingency is established during a Pavlovian threat conditioning session. In a separate session, the CS is presented alone and the experimental subject provided with the opportunity to

terminate the CS by performing a shuttling or another response like for example rearing. Experimental rats can learn to avoid in this paradigm, albeit less well than in the two-way shuttlebox.[Amorapanth et al., 2000; Cain and Ledoux, 2007; LeDoux et al., 2017] In addition to the described two stages of active avoidance learning, LeDoux and Cain highlight the importance of habit formation in 2wAA. Upon extensive learning, the performance of avoidance seems to become independent of reinforcers and resistant to extinction. [LeDoux et al., 2017]

In summary, avoidance is an important part of the rodent and human defensive system. How animals learn to avoid is not completely clear, however it seems likely that the environmental cues (CS) are associated with an US via a Pavlovian threat conditioning. Subsequently, an instrumental learning takes place which enables the animal to avoid a the application of the US. A number of potential (negative) reinforcers exist, and it is likely that several of them act concurrently to establish effective performance in an active avoidance task. Another interesting field of study is the delineation of neural circuits involved in learning and performance of active avoidance.

1.8 Neuronal basis of defensive behaviours in rodents

The amygdaloid complex has been discovered in human brain tissue by the german anatomist Karl Friedrich Burdach, who first published his findings in 1819. The term was chosen to describe an almond-like shape of a structure located deep in the medial temporal lobe. More than a century later, in 1937, first hints about the function of the amygdaloid complex were gathered by Klüver and Bucy. They studied monkeys with extensive damage of their medial temporal lobes and observed that the subjects had a range of behavioural peculiarities, including blunted or completely absent defensive and aggressive behaviours. [Davis and Whalen, 2001] In the 1950s, targeted stimulations and lesions of the amygdala were performed in monkeys. These studies found that stimulations of the amygdala elicited defensive reactions [Delgado et al., 1956], whereas lesions made the monkeys less likely to exhibit defensive reactions. [Weiskrantz, 1956] In fact, the stimulation experiments were performed on monkeys trained with an active avoidance task in which they had to pull and overturn a cup in response to the conditioned auditory stimulus. Electrical stimulation caused conditioned avoidance responses to be displayed in absence of a CS. [Delgado et al., 1956] In the Weiskrantz study, the amygdala lesioned monkeys trained in an avoidance task showed a faster extinction rate (and likely also slower acquisition of the avoidance task) than sham operated controls.[Weiskrantz,

1956] Conclusions drawn from observation of human patients with the Urbach-Wiethe syndrom follow a similar logic. These patients have lesions of the medial temporal lobes that arise as a result of tissue calcification, and they display a range of emotional problems that include impaired recognition of fearful facial expressions and impaired processing of emotional memories.[Adolphs et al., 1994, 1995, 1997] Nowadays, most detailed studies about amygdala function in threat processing and conditioning come from studies with rodents. [Calhoon and Tye, 2015; Davis and Whalen, 2001; LeDoux, 2000; Tovote et al., 2015] Other brain structures involved in coordinating the execution and memory within the threat defence system are often found to be anatomically highly interconnected with the amygdala. Instead of depending on a single brain structure, the defence system seems to rely on a distributed network that involves many different brain sites and very diverse types of neurons. The amygdala is not only connected with the cortical and thalamic sensory systems that process and relay incoming auditory, visual and olfactory information, but also with nucleus accumbens, hippocampus, higher associative cortices like the medial prefrontal cortex (mPFC), and also with evolutionary older hypothalamic and brainstem regions like the periaqueductal gray (PAG), parabrachial nucleus, and the dorsal vagal complex.[Calhoon and Tye, 2015] Importantly, some of the functionally relevant connections are anatomically indirect.

1.8.1 General structure of the amygdaloid complex

Amygdala is evolutionarily a relatively old part of the brain. Amygdala-like structures have been found in amphibians and other lower vertebrates. [Moreno and González, 2005] In 1923 Johnston was the first to subdivide the amygdala complex into central, medial, cortical and basal nuclei. This partitioning has been further refined in the course of the 20th century based on cyto- and chemoarchitectural details. However, there is still no consensus on the neuroanatomical description of the amygdaloid complex.[Alheid, 2003; Swanson and Petrovich, 1998]. Depending on the anatomical markers used, one can come to different conclusions about how to properly subdivide the amygdala into substructures. Instead of a subdivision based on anatomical markers, he proposed a division into four functional systems [3] : the frontotemporal, autonomic, main olfactory, and accessory olfactory systems. One of the less disputed is the division of the amygdaloid

[3] "The amygdala is neither a structural nor a functional unit of the cerebral hemispheres; instead, its cell groups participate in (...) distinct, though interconnected, functional systems or differentiations of the corticostriatopallidal system. Terms such as 'amygdala' (...) combine cell groups arbitrarily rather than according to the structural and functional units to which they now seem to belong.' [Swanson, 2003]

complex into two groups of nuclei depending on their embryological origin. The pallial amygdala develops mostly from derivates of the ventral and lateral pallium and is cortex-like, whereas subpallial amygdala shares its origins with striatum and pallidum.[Soma et al., 2009] The division into different classes based on cyto- and chemoarchitectural properties is very similar, as nuclei with shared embryonic origin share similar features. A classification based on McDonald's work is widely accepted in its gross outlines. [McDonald, 1998] According to this classification, the amygdaloid complex is divided into the basolateral group, the corticomedial group, and the centromedial group. The basolateral group consits of the lateral (LA) and basal (BA) nuclei, which are often referred to as the basolateral amygdala (BLA). Additionally, the accessory basal nucleus belongs to this group as well. The corticomedial group is located at the surface of the brain and their "nuclei" have layered structure not unlike that of the cortex. Both the corticomedial and basolateral group belong to the pallial amygdala. The centromedial nuclei are of subpallial origin and consist of the central (CEA) and medial nuclei, as well as the amygdaloid part of the bed nucleus of stria terminalis (BNST). Intercalated nuclei (IC), anterior amygdala area and the amygdalo-hippocampal area cannot be assigned to either of the three groups due to very different features and are thus usually listed separately. [Sah et al., 2003] Each of the nuclei listed can be further divided into subnuclei, and they will be described in more detail later on only if directly relevant to the project.

1.8.2 Basolateral amygdala

Up to 80% of BLA neurons are principal neurons (PNs), that is, glutamatergic spiny projection neurons. They arborise locally, but also send their axon collarterals to other amygdala nuclei as well to long-range targets. [Carlsen, 1988; McDonald, 1982a; Sah et al., 2003] In fact, the PNs are often grouped into more or less coherent functional classes based on their projection targets. [Herry et al., 2008; Senn et al., 2014] The non-glutamatergic 20% are GABAergic, aspiny interneurons (INs) that mainly arborise locally, and connect to both PNs and other INs. [McDonald, 1982a; Sah et al., 2003; Wolff et al., 2014] The INs are a very heterogenous group that can also be divided into several classes based on their morphology, electrophysiological properties or expression of genetic markers. Commonly, they are grouped based on the expression of Ca^{2+} binding proteins or neuropeptides. [Ehrlich et al., 2009; Pape and Pare, 2010; Spampanato et al., 2011; Wolff et al., 2014] The BLA receives a plethora of sensory information via direct thalamic and indirect thalamo-cortical routes. Virtually all sensory cortices send direct projections to the BLA and particularly the LA, and these cortical axons enter the amyg-

dala mainly through the external capsule. [LeDoux et al., 1990; Sah et al., 2003] The direct thalamic inputs stem from sensory processing areas of the thalamus which enter the LA via the internal capsule.[LeDoux et al., 1990]. These sensory inputs are thought to provide information necessary to asses threat levels and associate them with sensory cues from the environment. Ex vivo and in vivo experiments have shown an increase in synaptic transmission onto PNs upon Pavlovian threat conditioning.[McKernan and Shinnick-Gallagher, 1997; Pape and Paré, 2010; Quirk et al., 1997; Rogan et al., 1997; Tsvetkov et al., 2002] Lesions of the BLA induced in rodents prior to olfactory Pavlovian threat conditioning result in impaired CS-US association.[Cousens and Otto, 1998] Transient muscimol-based BLA inactivations reduce the strength of CS-US association without affecting the consolidation process. [Wilensky et al., 2006] Inactivation after fear conditioning leads to impaired retrieval and extinction of the threat memory. [Cousens and Otto, 1998; Herry et al., 2008] Interestingly, studies using in vivo recordings during Pavlovian threat conditioning found that a population of BLA neurons exists that is responsive specifically to the CS paired with US (CS+), but not to the unpaired CS (CS-). These neurons were found in in cats, rats and mice. Furthermore, two populations were found to emerge during conditioning: the "fear neurons" selectively increase firing to the CS+ only after FC, but return to baseline after extinction. On contrary, the population of "extinction neurons" increases their firing only after extinction. [An et al., 2012; Collins and Pare, 2000; Ghosh and Chattarji, 2015; Herry et al., 2008] The origin of the observed, NMDA receptor dependent plasticity is thought to be found on thalamo-LA, rather than on indirect thalamo-cortical-LA synapses. This is on one hand based on the observation that upon threat conditioning plasticity[Goosens and Maren, 2004] is observed in the LA prior to the cortical sites. On the other hand, it is the short latency of CS responses, which is thought to be the signature of thalamo-LA synapses that is potentiated. [Collins and Pare, 2000; Quirk et al., 1995] Inhibitory transmission, carried by BLA INs, is thought to coordinate and fine tune the process of acquisition and expression of threat memories. [Ehrlich et al., 2009; Tovote et al., 2015; Wolff et al., 2014] Apart from sensory inputs, the BLA is in fact heavily interconnected with polymodal associative brain areas like the mPFC, and hippocampal formation (HC). Processing in the HC is important for formation of contextual memories. In fact, ventral hippocampus inputs to the BLA are known to be important in retrieval of contextual memories, whereas mPFC plays a role in behavioural flexibility, for example transitions between behavioural states of no freezing and freezing. [Ciocchi et al., 2015; Corcoran and Maren, 2001; Dejean et al., 2016; Herry et al., 2008; Karalis et al., 2016; Orsini et al., 2011; Senn et al., 2014; Xu et al., 2016] The BLA principal neurons have projections to other nuclei

of the amygdaloid complex.

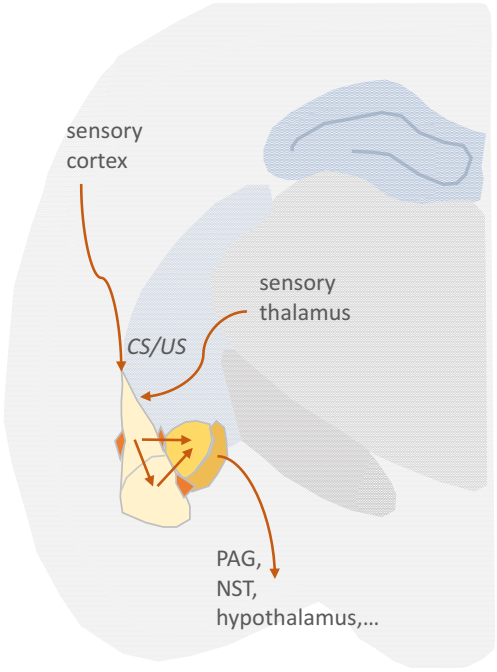

Figure 1.4: Classical view of information flow during Pavlovian threat conditioning

For example, the LA is known to project to the BA, and both LA and BA can project to the CEA. More precisely, the central lateral amygdala (CEl) is thought to receive inputs preferentially from the LA, whereas BA projects to both the CEl and the centromedial nucleus CEm.[Pitkänen et al., 1995; Savander et al., 1996] The BLA to CEA projections are known to have the potential to bidirectionally influence behaviour: mice show less thigmotaxis and spend more time in open arms of an elevated plus maze when this projection is activated. When inactivated, animals' behaviour resembles more closely that of an anxiety-like state.[Tye et al., 2011]

In summary, according to the classical view of information flow during Pavlovian threat conditioning, sensory information about the CS and US converge in the BLA. This convergence potentiates thalamic and cortical synapses relaying CS information onto LA neurons. This allows future CS presentation to elicit larger responses in LA neurons and to set off defensive reactions without the presence of a US. This is achieved mainly via projection to the CEA, which in turn coordinates defence via its projections to the hypothalamus and various brain stem sites. Additionally, BA also receives and further processes incoming sensory information from the LA, together with inputs from higher

associative cortices, allowing for fine tuning and flexibility of defensive responses, again via its projections to the CEA. [Herry and Johansen, 2014; Pape and Pare, 2010; Pare, 2004]

1.8.3 Central amygdala

Central amygdala is classically considered to be the output station of the amygdaloid complex that converts inputs from the BLA into outputs sent to target regions involved in execution of behavioural and autonomic correlates of threat states. [Ehrlich et al., 2009] Rather than being a simple relay, the CEA has an active role in formation and retrieval of threat-related memories. [Samson, 2005] First indications for a possible role of the central nucleus in threat memory formation or expression come from neurotoxic lesions that attenuate conditioned defensive reactions to both auditory and context cues. [Goosens and Maren, 2001] Further evidence comes from studies in which the functioning of the CEA is transiently modified by either reversible muscimol based inactivation, by NMDA receptor blockage, or by inhibition of protein synthesis. [Ciocchi et al., 2010; Goosens and Maren, 2003; Wilensky et al., 2006; Zimmerman et al., 2007] Interestingly, although BLA pre-training lesions result in poor threat memory, this effect can be overcome by overtraining. This overtraining effect is thought to be mediated by the CEA [Zimmerman et al., 2007].

Apart from its role in conditioned defence, the CEA can control the expression of unconditioned defensive behaviour. CEl inactivation with muscimol in naive rodents is known to induce unconditioned freezing. [Ciocchi et al., 2010] Furthermore, the CEA is thought to play a role in risk-assessment behaviours. Electrolytic lesions of the CEA decrease anxiety-like behaviours, for example increased thigmotaxis can be observed in the open field test.[Jellestad et al., 1986] New studies show that inactivation of BLA to CEA inputs also promotes an anxiety-like behaviour in the open field and plus maze. [Tye et al., 2011] Furthermore, tonic firing changes in genetically defined populations of neurons is causally linked to these behavioural changes. [Botta et al., 2015]

Based on cyto- and chemoarchitecture, the CEA can be subdivided into the centrolateral (CEl), and the centromedial (CEM) amygdala. Additionally, the lateral part is often subdivided into lateral and capsular-lateral (CElc) divisions. As expected for structures of subpallial origin, and similarly to the striatum, the CEA consists of 90% GABAergic, mostly medium-spiny neurons.[Cassell et al., 1999; Ehrlich et al., 2009; Haubensak et al., 2010; McDonald, 1982b; Sun and Cassell, 1993] Neurons of the CEA are known to express a diverse set of neuropeptides and neuromodulatory receptors, including SOM [Butler

et al., 2012; Moga and Gray, 1985; Sajdyk et al., 2004; Wray and Hoffman, 1983], corticotropin releasing factor (CRF) and CRF receptors[Heinrichs et al., 1992; Merchenthaler et al., 1982; Radulovic et al., 1998; Reyes et al., 2016; Yu and Shinnick-Gallagher, 1998], opioid peptides dynorphin and enkephalin[Cassell and Gray, 1989; Haubensak et al., 2010; Watson et al., 1982], as well as delta-, kappa- and mu- opioid receptors[Chieng et al., 2006; Zhu and Pan, 2005], oxytocin and oxytocin receptor, as well as vasopressin and vasopressin receptor[Veinante and Freund-Mercier, 1997], glucocorticoid receptor, calcitonin-gene related (CGRP), galanin[Puskas et al., 2007; Skofitsch and Jacobowitz, 1985], substance P[Roberts et al., 1982], neurotensin, cholecystokinin [Roberts et al., 1982], and others. Although a lot of work was done on the chemo- and cytoarchitecture of the peptidergic system within the CEA, the roles of these peptides in controlling defensive reactions and threat memory have still not been explored in detail and their action on amygdala microcircuits is pretty much unknown.

Connectivity within the CEA is observed among neurons of each subdivision, but also from neurons located in the CEl to CEm neurons. A connection from CEm to CEl has not been observed so far, although CEm seems to provide a sparse input to the CElc.[Jolkkonen and Pitkänen, 1998] Although the CEm neurons are the ones that are usually referred to as the output of the amygdaloid complex, CEl neurons are also known to cross CEA borders and project for example to the BNST, hypothalamus, PAG and the parabrachial nucleus (PB). [Cai et al., 2014; Fadok et al., 2017; Tovote et al., 2016; Veinante and Freund-Mercier, 1997]

Extrinsic inputs to the CEA come for example from glutamatergic neurons from the BLA. It is known that LA and BA inputs to the CEA are potentiated upon Pavlovian threat learning, and that activity of the neurons with potentiated synapses is important for this type of learning. [Li et al., 2013; Watabe et al., 2013] A bidirectional connection with the dorsal midline thalamus and paraventricular thalamus has also been observed. PVT neurons send an excitatory input to PKCδ^+ and SOM$^+$ neurons in the CEA. In fact, the increase in excitatory synaptic strength of LA onto SOM$^+$ neurons observed 24h after Pavlovian threat conditioning is thought to be mediated by the PVT projection to the CEA.[Do-Monte et al., 2015; Penzo et al., 2015] The CEA, and especially its medial nuclus also receive inputs from other thalamic regions that are thought to convey sensory information, for example from the posterior thalamic nuclei. [LeDoux et al., 1985] In fact, in vitro patch experiments indicate that stimulation of the external capsule that contains thalamic inputs to the amygdala can activate a different set of CEm neurons than BA stimulation. Furthermore high frequency stimulation of these inputs leads to long-term potentiation of excitatory transmission onto CEm neurons,

in a LA independant manner. This is an indication that sensory inputs coming from posterior thalamic nuclei have plastic synapses - a property that might be exploited to contribute to threat learning [Samson, 2005]

Neuromodulatory and neuropeptidergic inputs to the CEA have also been observed. Serotonergic inputs, as well as a number of CEA neurons expressing serotonergic receptors have been verified experimentally. [Chalmers and Watson, 1991; Isosaka et al., 2015; Linley et al., 2017; Sun et al., 2015] Strong noradrenergic innervation and expression of noradrenergic receptors have also been observed. [Delaney et al., 2007; Fallon et al., 1978; Talley et al., 1996] Furthermore, there is a prominent dopaminergic innervation, and the activity of D2 receptors is known to be important during Pavlovian threat learning. [De Bundel et al., 2016; Guarraci et al., 2000; Hasue and Shammah Lagnado, 2002; Smith et al., 2015]. In fact, most of D2 receptor expressing neurons are also positive for protein kinase C delta (PKCδ^+). [De Bundel et al., 2016]

PKCδ is thought to be a good marker for a functionally distinct group of neurons that decrease their phasic firing to the CS upon Pavlovian threat conditioning (figure 1.5). [Haubensak et al., 2010]

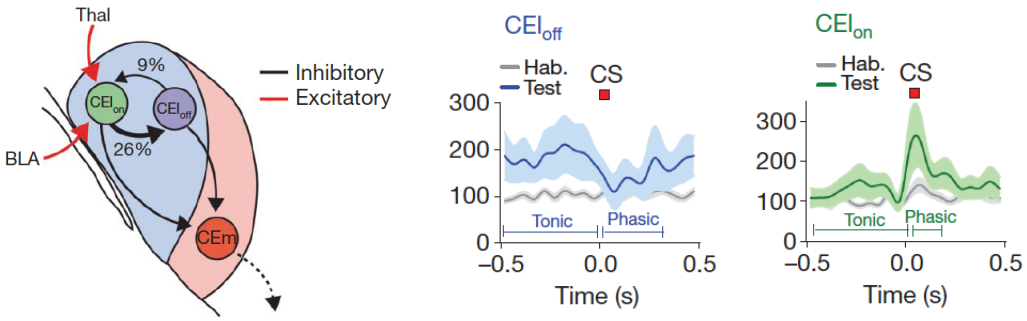

Figure 1.5: Three functional classes of CEA neurons emerge upon Pavlovian threat conditioning. Adapted from Ciocchi et al. [2010].

This functionally distinct class is also known as CEl$_{off}$ neurons. Although we know that all CEl$_{off}$ neurons express PKCδ, we do not know for sure what percentage of PKCδ^+ neurons are CEl$_{off}$. Since PKCδ^+ neurons seem to be slightly more numerous than CEl$_{off}$ neurons, there are likely to be some PKCδ^+ neurons that do not belong to this functional class.[Ciocchi et al., 2010; Haubensak et al., 2010] CEl$_{off}$ neurons do not only change their phasic, but also their tonic firing after Pavlovian threat conditioning - namely in the

opposite direction. Whereas their phasic activity is negatively correlated with freezing behaviour, their tonic activity is positively correlated with it. Furthermore, the change in tonic activity (as compared to freezing) was found to be significantly higher in experimental subjects that generalise between CS paired with US, also called CS^+ and the unpaired CS^-. This study also discovered another population of neurons in the CEl that increases phasic firing to the CS^+ upon Pavlovian threat conditioning. These so-called CEl_{on} neurons seem not to display changes in tonic firing. Lastly, the recorded CEm neurons all exibited an increase in phasic firing to CS, and a decreased tonic activity. It is postulated that the phasic firing of CEm neurons is result of a fast onset excitation (thalamic component) and an increase in activity with a slower time course that likely stems from disinhibition by CEl_{off} neurons.[Ciocchi et al., 2010]
The observed increase in tonic firing is thought to be due to decreased GABAergic transmission specifically via extrasynaptic α5-subunit containing $GABA_A$ receptors on $PKC\delta^+$ neurons.[Botta et al., 2015] Another interesting aspect of $PKC\delta^+$ neurons is that they can increase their activity (as assessed by expression of the immediate early gene cfos) in response to anorexigenic challenge. In fact, inactivation of these neurons can cause an increase in food intake, whereas their activation inhibits feeding. The study by Cai et al. finds anxiolytic-like effects of optogenetic activation of $PKC\delta^+$ neurons. This observation is in contrast to the study by Botta et al, and these divergent results might be due to very different stimulation parameters used in the two studies. [Botta et al., 2015; Cai et al., 2014] These differences might point in the direction of multi-purpose circuits within the central amygdala in which different firing rates code for different outcomes in terms of behaviour.
Yet another aspect of CEA function is its role in threat-related analgesia, which is thought to be induced in CEA and executed in the PAG and the downstream ventromedial medulla in a opioid dependant manner.[Fields, 2004; Oliveira and Prado, 2001; Pan et al., 1997; Rizvi et al., 1991] Interestingly, the antinociceptive effect induced by morphine is attenuated in CEA lesioned experimental subjects.[Manning and Mayer, 1995]. The CEA also seems to play a role in mediating behavioural and motivational effects of rewards.[Baxter and Murray, 2002; Gallagher et al., 1990]Reinforcing effects of durgs, for example alcohol and opioids, are also thought to be mediated by the CEA. Very recent evidence suggests that the CEA also plays a role in predatory hunting via CEm efferents to vlPAG and the reticular formation. [Han et al., 2017]

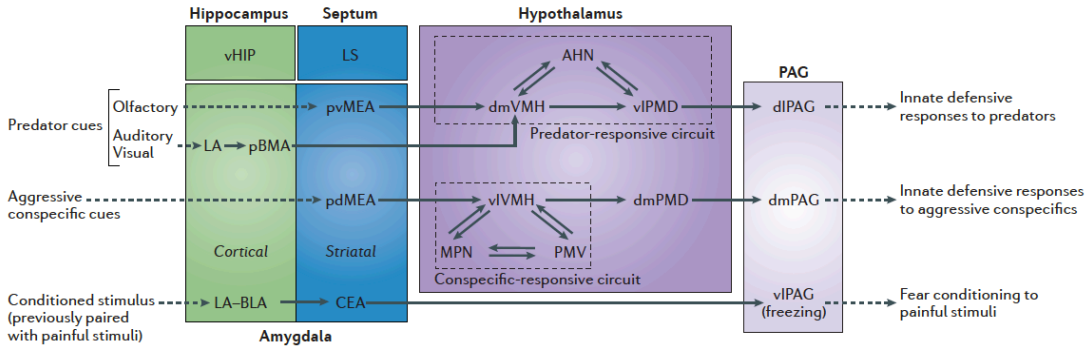

Figure 1.6: Distributed, parallel defensive systems. Adapted from Gross and Canteras [2012].

1.8.4 Parallel distributed defensive systems

As mentioned above, the BLA relies on its connections to other brain areas for adequate coordination of defensive behavioural reactions to threats. Thalamic and cortical inputs, as well as some brainstem inputs provide sensory information about threats. Hippocampal inputs yield information about the context in which the threat occurs, and mPFC input is important for processing information related to behavioural flexibility needed to reevaluate stimuli in face of novel circumstances. Outputs to brainstem areas like the PAG are needed for initiating freezing and flight, and for coordinating activity in premotor areas. The latter is in turn needed to adequately control muscle actions necessary for the execution of defensive behaviours. Autonomic and hormonal reactions to threats are coordinated via several areas in the brain stem that CEA projects to - for example via PAG, nucleus tractus solitarius, PB, LC and hypothalamus. Additionally, the neuronal systems involved in threat processing are thought to be not only distributed, but also operating in parallel to each other. According to the model suggested by Gross and Canteras, processing of predator and conspecific threats is processed in separate circuits, depending on whether visual, auditory or olfactory cues need to be processed. The amygdala is still placed in the center of circuits coordinating associative learning that involves auditory and visual cues in addition to painful stimuli. This pathway does not engage the hypothalamic circuits to the same extent as predator and conspecific threats do. In fact, processing of olfactory cues might largely circumvent the BLA and instead rely on communication between the medial amygdala and the hypothalamus, as well as downstream brainstem areas. [Gross and Canteras, 2012; Kunwar et al., 2015] Interestingly, these pathways are suggested to primarily communicate with different columns of the PAG. Early lesion and excitatory amino acid infusion studies have found

that different columns of the PAG are responsible for different defensive behaviours. According to this model, dorso- lateral PAG (dlPAG) coordinates active defence and can induce non-opioid mediated analgesia, hypertension and tachycardia. More precisely, the rostral extent of the dlPAG is responsible for coordinating defensive aggression, whereas its rostral parts are mainly associated with flight. The ventro-lateral PAG (vlPAG) is associated with passive defence, but also with vocalisation, bradycardia, hypotension and opioid mediated analgesia.[Bandler et al., 2000a] A recent study by Tovote et al. was the first to explore how genetically distinct populations of neurons in this column control freezing and analgesia. Activation of vlPAG glutamatergic cells above baseline activity levels with optogenetic methods, caused a rapid onset of freezing, whereas their inhibition lowered immobility in response to both innate and conditioned threats. It appears that specifically the glutamatergic vlPAG cells projecting to the magnocellular nucleus of the medulla, a premotor area of the brain, are responsible for this effect. On the other hand, GABAergic neurons of the vlPAG had the opposite effect on freezing behaviour. Ventro-lateral PAG is known to receive direct inputs from both the CEl and CEm. These inputs might provide one source of control over whether these defensive behaviours would be activated.[Rizvi et al., 1991; Tovote et al., 2015] The dlPAG might be especially responsive to predator threats during which it controls defensive aggression, in addition to flight behaviour, hypertension, tachycardia and non-opiod mediated analgesia. In fact, activation of glutamatergic cells in the l/dlPAG is known to induce unconditioned flight behavoiour.[Bandler et al., 2000a; Carvalho-Netto et al., 2006; Tovote et al., 2015].

The prefrontal cortex is known to be involved in expression of defensive behaviours. It has synaptic partners in the amygdala, but also sends direct projections to the PAG, both of which are thought to be effector targets for fear expression. [Courtin et al., 2013; Sesack et al., 1989; Vertes, 2004] An intriguing new phenomenon, LFP-phase specific coding of behaviour has been observed in the mPFC, in which principle neurons form functional assemblies with synchronised enhancement in firing at the onset of freezing and the coincident emergence of theta oscillations. Optogenetic inhibition of principle neurons during the ascending phase of a theta cycle results in decreased freezing, whereas inhibition during the descending phase results in enhanced freezing.[Dejean et al., 2016] The prefrontal corex seems to also contain "strategy selective" neurons, that are preferentially activated either during conditioned passive or active defence. Similarly, active defence specific neurons are also found in the dlPAG. [Halladay and Blair, 2015] The mPFC layer 5 neurons have recently been found to project to the dorsal PAG (dPAG) and are thought to synapse onto glutamatergic cells there. Interestingly, inhibition of

dPAG glutamatergic neurons was found to be a potent inhibitor of social avoidance to a conspecific aggressor.[Franklin et al., 2017]

1.9 Neuronal basis of active avoidance in rodents

As mentioned previously, the rodent brain seems to process threat stimuli in parallel and distributed neuronal circuits. The bulk of current research on threat processing was performed using passive defence, and specifically conditioned freezing as a behavioural model. However, active defence is again increasingly getting into the focus of neuroscience research. Although a plethora of research has been done on characterising the learning processes and the behaviour of rodents undergoing active avoidance conditioning, surprisingly little is known about the neuronal circuits involved in controlling the learning and expression of this behaviour.
Several studies explored the activation patterns in different brain regions with the purpose of gaining overview of brain regions involved in active avoidance. Study of CREB expression in the brain followng two-way active avoidance conditioning in rats has found increased numbers of cells with phosphorylated CREB in the BLA, CEA, hypothalamus and hippocampus.[Saha and Datta, 2005] Another study searched for increased fMRI signals following pharmacogenetic inhibition of the central amygdala, a manipulation that results in increased conditioned active defensive behaviours, and found an activation of the basal forebrain and the cortex.[Gozzi et al., 2010] All these brain areas have been confirmed to be involved in active defence with lesion, inactivation or activation studies. For example, intracranial stimulation of the lateral hypothalamus results in enhanced 2wAA [Kadar et al., 2010], whereas lesions of the nucleus basalis have yielded conflicting information. It seems that they influence long-term retention but not necessarily learning of 2wAA.[Vale-Martínez et al., 2002] Gross hippocampal lesions were found to facilitate avoidance.[Guillazo-Blanch et al., 2002; Olton and Isaacson, 1968] It was hypothesised that this is because the hippocampus is involved in forming a memory about the context in which the US occurred. Since in 2wAA the shock occurs in both parts of the context, the hippocampus might be signalling that the context beyond the divider is dangerous as well, thereby counter-acting efforts to avoid the upcoming shock. Finer-grained inactivations with muscimol indicate that inactivation of either ventral or dorsal hippocampus can impair performance in the 2wAA task.[Wang et al., 2015] Cortical regions implicated in avoidance are for example the anterior cingulate cortex (ACC) and the mPFC. The ACC has been implicated in avoidance of dynamic, visual

CS in rats.[Svoboda et al., 2017] As the medial prefrontal cortex has a well established link to learned passive defensive behaviours, it is perhaps not surprising that it was also found to play a role in active avoidance. Pre-training muscimol- based inactivation of the ilPFC was found to enhance conditioned freezing and lower the conditioned avoidance in a 2wAA task. Intriguingly however, inactivation of the plPFC did not result in changed performance in the 2wAA task.[Moscarello and LeDoux, 2013]

The neuromodulatory pathways seem to have a very strong link with active avoidance. Dopamine deficiency is accompanied by a plethora of behavioural defects and was also found to result in impaired 2wAA performance.[Kobayashi and Sano, 2000] Deep brain stimulation of the ventral tegmental area (VTA), which is thought to increase dopamine in the system, is a potent enhancer of active avoidance [Ilango et al., 2011]. It seems that dopamine signalling is particularly important in the nucleus accumbens (NAc) as both pre- and post-training infusions of D2 agonists in the NAc result in impaired active avoidance.[Boschen et al., 2011] Furthermore, dopamine signalling in both striatum and the BLA is necessary for optimal 2wAA.[Darvas et al., 2011] Another study by Dombrowski et al. has explored dopamine release in rat striatum during two-way active avoidance training. They find unchanged striatal dopamine levels in animals that experienced unpredictable and inescapable US, but observe strong dopamine peaks during normal 2wAA training trials. These peaks are highest at the beginning of training and progressively decrease over time. The authors conclude that this might be an indication that successful, unexpected avoidance generates a positive prediction error that drives avoidance learning. The decrease of dopamine levels back to baseline correlates with improved avoidance learning and is consistent with a decreased prediction error.[Dombrowski et al., 2013] Further evidence in support of striatal involvment comes from selective deletion of striatal adenosine 2A receptors which results in impaired performance in the 2wAA task. [Singer et al., 2013] The serotonergic system might also be involved in 2wAA. High performance in active avoidance tasks is associated with lower levels of serotonin in hippocampus and hypothalamus, compared to levels found in low performance experimental subjects.[Sfikakis et al., 2002] On the other hand, systemic injections of serotonin receptor agonists can enhance active avoidance learning.[Alhaider et al., 1993]

The involvement of the amygdala in different types of active avoidance paradigms was explored already in the 1950s in studies with monkeys.[Delgado et al., 1956; Weiskrantz, 1956] Several studies in cats and dogs found similar effects, thus providing strong hints that the role of amygdala in active avoidance is likely a conserved feature in mammals, and that both BLA and CEA are involved.[Brady et al., 1954; Fonberg, 1965; Horvath,

1963; Ursin, 1965; Werka and Zieliński, 1978] Post-training electrolytic amygdala lesions in rats have also been found to cause impairments in active avoidance. The lesions did not have any effect on avoidance performance in overtrained rats, yielding one of the first hints that habitual avoidance is amygdala independant. [Thatcher and Kimble, 1966] Interestingly, some studies found that amygdala lesions result in enhanced performance in a two-way active avoidance task[Grossman et al., 1975; Kemble and Davies, 1981], whereas others did not find an effect at all.[McNew and Thompson, 1966] These confusing and partially contradicting evidence might be due to different lesion techniques, time points of introducing the lesions, or due to usage of slightly different avoidance paradigms. Small changes in any of these parameters could lead to large differences in observed effects. In mice, electrolytic lesions of the amygdala were found to impair acquisition of active avoidance in the 2wAA task.[Takashina et al., 1995] Precise pre-training lesions of amygdala nuclei in rats trained in the escape from fear (EFF) paradigm reveal that LA and BA lesions, but not CEA lesions impair performance in the EFF task.[Amorapanth et al., 2000] Fiber sparing NMDA lesions of BLA, but not CEA was found to impair the acquisition of 2wAA in mice. However, if CEA lesions were performed after training in experimental subjects that did not learn the task, further training allowed the lesioned, but not sham operated subjects to learn the task. Learning curve in lesioned non-learners was steeper, but saturated at the same levels as training of high-avoiders that were sham-operated.[Choi et al., 2010] This indicates that the CEA in these animals placed a break on the avoidance learning circuit for largely unexplored reasons. Interestingly, similar results were found in rats trained in a Sidman avoidance task. However, in this case the CEA lesions in poor performers resulted in asymptotic avoidance during the first session, leading the authors to contemplate the possibility that the animals have in fact learned the task, but that the activity in the CEA was inhibiting its expression because of excessive freezing.[Lázaro-Muñoz et al., 2010] Yet another study that helped consolidate the role of CEA in active avoidance was published in 2013 by Moscarello and coworkers. Pre-training muscimol inactivations of the CEA resulted in lower avoidance rate on the day of the muscimol infusion, while at the same time increasing conditioned freezing. This impairment was not evident on subsequent conditioning days. However, when the authors infused protein synthesis inhibitor anisomycin after the first training session, they could observe improved consolidation of 2wAA, evident as ameliorated responding in the 2wAA task on the next training day. [Moscarello and LeDoux, 2013]

Cellular and circuit substrates of the CEA role in active avoidance are only starting to be explored. In 2010 Gozzi and coworkers explored how the behaviour of mice is

influenced by inhibition of "Type I" cells in the central amygdala. These neurons are likely PKCδ$^-$ and have a characteristic depolarising current following action potentials. Pharmacogenetic inhibition of Type I cells resulted in an increase in risk assessment and exploratory behaviours during the presentation of a CS, which the authors summarise under the name "active behaviours". The inhibition of these Type I cells also resulted in an increased blood flow in amygdala, basal forebrain and the cortex, as assessed with fMRI. Furthermore, they tonically suppress PKCδ$^+$ cells, which are the proposed "Type II" effector cells. Type II cells are postulated to mediate CEA output to both CEm brainstem projectors and to cholinergic centers in the nucleus basalis. The disinhibition of the cholinergic system achieved with this supression of Type II cells is suggested to result in higher cortical arousal, and thus an increase in active defensive behaviours. [Gozzi et al., 2010]

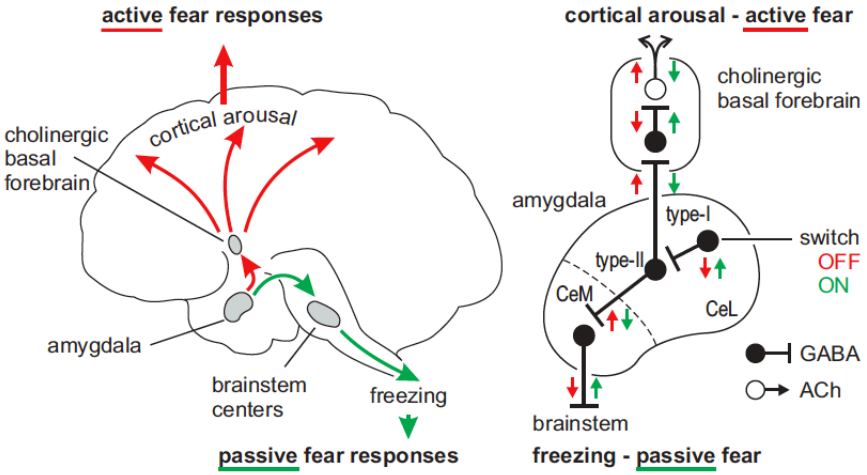

Figure 1.7: A model of CEA's role in active defensive behaviours according to Gozzi et al. [2010]. Adapted from Pape [2010].

Two very recent studies have found that different types of PKCδ$^-$ neurons are implicated in active defence.[Fadok et al., 2017; Yu et al., 2016] The SOM$^+$ neurons in the CEA are known to be capable of inducing freezing [Li et al., 2013] and have now been assigned a role in active avoidance as well. The authors of the new study trained head-fixed mice in a modified active avoidance paradigm and found that acute optogenetic activation of SOM$^+$ neurons inhibited the performance of conditioned avoidance responses.[Yu et al., 2016] This is likely an indirect effect of increased freezing that is known to occur upon activation of SOM$^+$ neurons. In another recent study, Fadok et al have developed a

new Pavlovian conditioning paradigm, that goes under the name of "conditioned flight". Conditioned flights results in increased active, instead of passive defence upon presentation of a conditioned stimulus. They find that optogenetic inhibition of SOM^+ neurons in this paradigm does not lead to effects on neither freezing, nor on flight, whereas their activation resulted in an increased freezing and decreased flight. On contrary, inhibition of CRF^+ neurons in the CEA lead to an increase in conditioned flight without affecting freezing behaviour. Activation of CRF^+ neurons decreased conditioned flight and led to an increase in freezing.[Fadok et al., 2017] Interestingly, the role of $PKC\delta^+$ neurons in active defence is still largely unexplored.

Aim of the thesis

Involvement of central amygdala PKCδ^+ neurons in active defence has not been extensively explored so far. They are an interesting candidate to study, because of the observed negative correlation of CS-evoked phasic activity of PKCδ^+ neurons and of CS-evoked freezing in Pavlovian fear conditioning. This is observed in parallel to the striking negative correlation of freezing and active defensive behaviours. All this provides hints that increased phasic activity of PKCδ^+ CEA neurons during CS presentation might contribute to the creation of favourable conditions for the expression of active defensive behaviours. On the other hand, increased tonic activity of PKCδ^+ neurons by optogenetic methods has been found to be a potent inducer of risk assessment behaviour[Botta et al., 2015]. This yields cues that these neurons might be in a unique position that could allow them to control a range of different defensive bahviours with a firing rate code. It remains to be explored what the endogenous activity patterns of these neurons look like during more active types of conditioned defensive behaviour, and also whether activation of PKCδ^+ neurons can facilitate conditioned active defence.

My thesis explores whether PKCδ^+ neurons play a role in conditioned active defensive behaviour, using two-way active avoidance as a conditioning paradigm.

Results

We first searched for a paradigm that enhances active responding in mice, while at the same time being compatible with optogenetics and commonly used techniques in neurophysiology. Sidman avoidance, escape form fear and two-way active avoidance largely meet these criteria. Auditory signalled two-way active avoidance is acquired relatively quickly by mice, it uses a clearly defined CS and requires the experimental subjects to performe a well defined active defensive response, namely the avoidance of the US by shuttling during the CS-only phase.
Since commercial 2wAA setups were found to be mostly incompatible with in-vivo optogenetics and electrophysiology (closed boxes without space for cables), and to come with rather unsatisfactory behavioural monitoring systems (low-resolution IR-beam based tracking), we decided to use a custom-made setup. A standard fear conditioning context was modified in order to accommodate two separate, but equal chambers. The two halves each contained independently controlled electric grid floors and were separated by a grey plexiglass divider. The width of the passageway in divider was chosen such that it would allow easy transition between the two halves, even when the subjects had somewhat bulky head-stages and cables mounted on their head-caps. In order to be able to acquire a behaviourally rich data set, we decided on using a video-tracking based system for the execution of the instrumental training protocol. As such a system did not exist at the time, we have collaborated with Biobserve (Biobserve, Bonn, Germany) in order to develop a system that meets our requirements.

The two-way active avoidance protocol applied in this study is a modified version of the classical fear conditioning. It uses a 5kHz tone of a maximum duration of 10sec as CS, and pairs it with an extremely mild 0.3mA AC escapable shock with a maximum duration of 5sec as US. The first 5sec of the tone are not paired with a US, thus the tone and shock overlap during the second half of the CS and co-terminate (figure 3.1 A). The training

routine is performed on five days, first of which is a habituation session on which no US is applied. On four remaining days the actual 2wAA training is performed, which consists of 51 trials equally spaced by a 45sec inter-trial interval (ITI). The application of both CS and US depends on the subject's behaviour. For more details, see the Introduction and Methods sections.

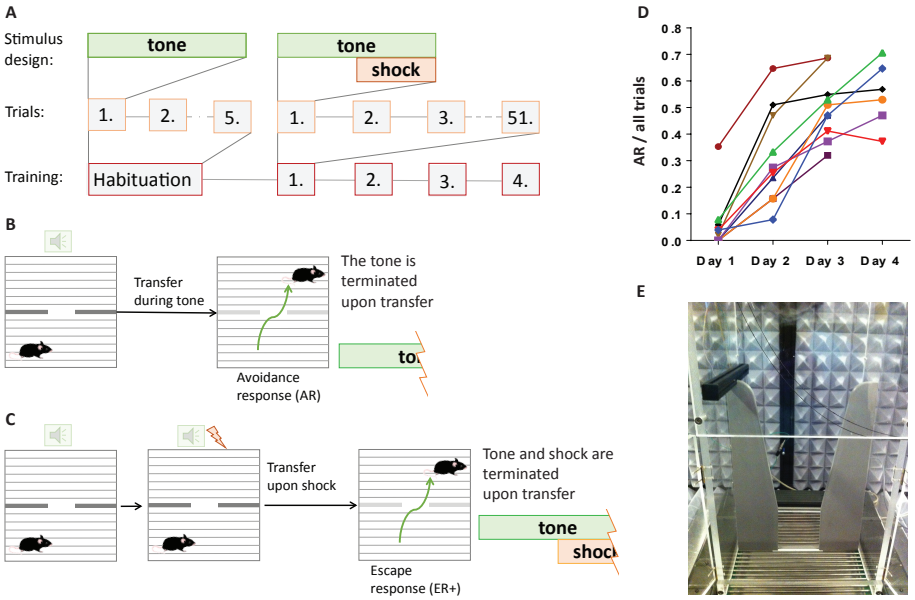

Figure 3.1: Two-way active avoidance. ***A*** Stimulus and trial design for the 2wAA protocol (for more details, see Methods section). ***B*** Animal behaviour and stimuli applied on avoidance response (AR) trials. ***C*** Animal behaviour and stimuli applied on successful escape response trials (ER+) ***D*** Emergence of avoidance responses (AR) during 2wAA conditioning. ***E*** Design of the 2wAA conditioning chamber.

Based on animals' behaviour, each one of the 51 trials was classified as an avoidance response (AR) trial, escape response (ER+) trial or a trial on which no shuttling was performed at all (ER-). If the subjects shuttle during the first 5sec of the CS, the CS is immediately terminated, the US is not applied, and the 45sec ITI is started. This type of response is called the avoidance response (AR), and the trials on which the behaviour was performed are respectively called AR trials. If the animals do not shuttle during the CS-only period, the shock is applied. At this point the animals exhibit an unconditioned active reaction which is characterised by a sharp increase in speed of motion (flight). If as a part of this unconditioned reaction (UR) the animal shuttles pass the divider and to the other side of the context during the CS, the CS and US are terminated immedi-

ately the trial is classified as a successful US-escape response trial (ER+). On very rare occasions, the animals do not perform a successful shuttle, thus being exposed to the CS and US for their maximum duration. Trials on which this happens are classified as unsuccessful escape response trials (ER-). Success of learning is directly correlated with the number of ARs performed (figure 3.1 D). Additionally, we used freezing and speed of motion in order to better characterise the active and passive defence strategies of the experimental subjects.
In order to test whether CEA neurons play a role in active defensive behaviour, we manipulated genetically defined populations of CEA neurons during two-way active avoidance training.

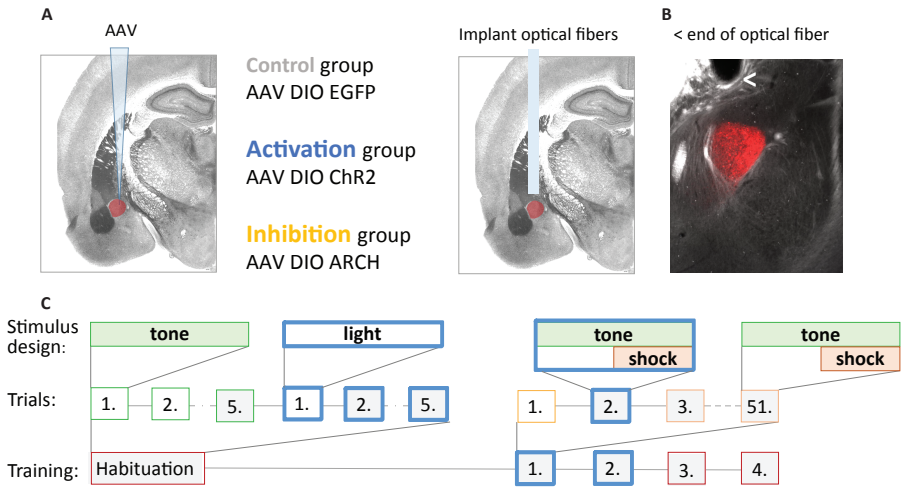

Figure 3.2: Optogenetic manipulations during two-way active avoidance. ***A*** Mice are bilaterally injected with one of the three rAAV and then implanted bilaterally with optical fibres (see Methods for further details). ***B*** Example of an optimal optic fibre implantation site. ***C*** Light manipulation protocol during 2wAA.

We injected rAAVs containing Cre- dependant constructs bilaterally into the CEA of either SOM-Cre or PKCδ-Cre transgenic mice. The constructs contained either Cre-dependant DIO.EGFP (control group), DIO.ChR2-eYFP (ChR group) or DIO.ARCH-GFP (ARCH group). Fiber- optic connectors were also implanted bilaterally just above the CEA in order to provide a path for optical manipulation of the virus-infected CEA neurons. The 2wAA paradigm was partially modified, in order to incorporate light-manipulation of CEA neurons at critical time points during learning and expression of active avoidance. As shown in figure 3.2 C, five light-only presentations were added at the end of the normal habituation protocol, in order to allow assessment of baseline

effects of light and light-based manipulation on the behaviour of experimental subjects. Further, during the first and second days of conditioning, light-based manipulation was applied on all even-numbered trials, starting with the second trial. The light was applied during the entire duration of the CS. All odd-number trials were normal, light-free trials. Comparison of animals' performance during even-numbered (light+) trials and during odd-numbered (light-) trials, allowed statements about acute effects of optogenetic manipulation of CEA neurons on expression of two-way active avoidance. Conditioning days three and four were entirely free of optogenetic manipulation. Comparison of AR-rates in control and opsin-groups on these two light-free days allowed statements about effects of optogenetic manipulation of CEA neurons on learning of 2wAA.

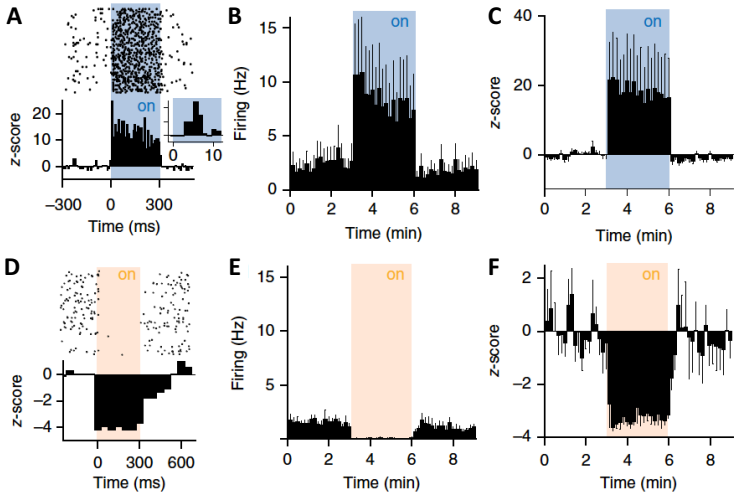

Figure 3.3: Effects of optogenetic manipulations of CEA PKCδ$^+$ neurons on their activity. Adapted from Botta et al. [2015]. ***A*** Opto-tagging experiments with ChR2-expressing PKCδ$^+$ units. Raster plot showing how a 300ms blue light pulse changes the firing of a PKCδ$^+$ neuron expressing ChR2. The light induced changes have a short latency (see inlet). ***B*** Average firing rate histogram of identified ChR2-expressing PKCδ$^+$ neurons before, during and after a three minute long blue light stimulation. ***C*** Average z-score histogram showing that blue-light induced changes in firing rate are highly significant (N = 5 cells from 2 mice). ***D*** Opto-tagging experiments with ARCH-expressing PKCδ$^+$ units. Raster plot showing how a 300ms blue light pulse changes the firing of a PKCδ$^+$ neuron expressing ARCH. ***E*** Average firing rate histogram of identified ARCH-expressing PKCδ$^+$ neurons before, during and after a three minute long yellow light stimulation. ***F*** Average z-score histogram showing that yellow-light induced changes in firing rate are highly significant (N = 6 cells from 3 mice).

In order to confirm that we are able to reliably induce significant changes in firing of PKCδ$^+$ neurons with the chosen optogenetic tools, we injected animals with the above

mentioned ARCH or ChR2 constructs and implanted them simultaneously with both optical fibres and with 16 low impedance extracellular single-unit recording electrodes. Then we applied 300ms pulses of blue or yellow light (see Methods) in order to identify short-latency responding neurons as PKCδ^+ (see Methods section for more details)[1]. After a successful identification of PKCδ^+ neurons, we applied three minute long constant blue or yellow light in order to confirm that we are able to activate/inhibit the opto-tagged PKCδ^+ neurons for longer durations. As evident from figure 3.3 B and C, we were able to significantly increase firing of the identified ChR2 expressing units for the entire duration of three minute long blue light application. As shown in figure 3.3 E and F, we were also able to significantly decrease firing of ARCH-expressing units for the duration of the three minutes yellow light pulse.

3.1 Optogenetic activation of PKCδ^+ neurons during 2wAA

PKCδ-Cre transgenic mice were injected either with AAV DIO ChR2 (ChR group) or with AAV DIO EGFP (control group) and implanted with optical fibers. After a minimum of five weeks after the AAV injection, we started the two-way active avoidance procedure, as described above. Although all implanted animals underwent the training, only animals with transgene expression restricted to the CEA and with fibers positioned up to 500μm away from the central nucleus were taken into the analysis. These were the only selection criteria applied and resulted in group sizes of N=10 for the control cohort, and N=10 for the ChR cohort. First we explored how the light and the manipulation of PKCδ^+ neurons affect locomotion of the experimental subjects. We found that light per se does not have an effect, whereas the manipulation of PKCδ^+ neurons does. Specifically, we observed rebound freezing, which started after the application of blue light through the optic connectors, specifically in the ChR, but not in the EGFP group (figure 3.4).

[1]The results of this experiment were published in Botta et al. [2015]. I have performed all ChR2-tagging experiments and analysis, while Jonathan Fadok has performed all ARCH-tagging experiments and analysis.

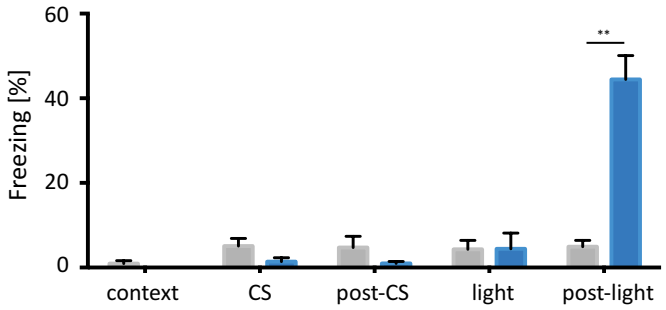

Figure 3.4: Baseline effects of ChR-based manipulation of PKCδ$^+$ neurons. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test.

A day after the habituation session, mice were subjected to the first day of 2wAA training on which blue light was applied on even-numbered trials only. We assessed how the ChR-based activation of CEA PKCδ$^+$ neurons affects performance of active avoidance. We find a significant increase in AR rate in ChR-group as compared to the control group on the first, and also all subsequent conditioning days (figure 3.5). As the difference persists also on the third and fourth conditioning days on which no optogenetic manipulations took place, we conclude that optogenetic activation of PKCδ$^+$ neurons boosts learning of 2wAA.

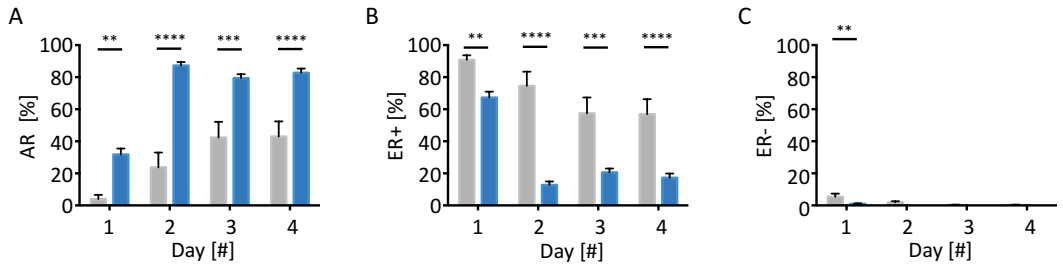

Figure 3.5: CEA PKCδ$^+$ neurons boost learning of active avoidance. Activation of PKCδ$^+$ ***A*** boosts AR, ***B*** decreases % ER+, and ***C*** ER- rates. Values indicate % of total trials). Control group is shown in grey, ChR group is in blue. Statistical tests: multiple unpaired paired t-tests, with Holm-Sidak correction for multiple comparissons.

In order to get a better overview of how the difference in AR rate develops and is maintained over time, we divided the 51 trials of the 2wAA protocol into three blocks of 17 trials each. This way, we observe that AR first becomes significantly higher on the second block of the first conditioning day (figure 3.6) and the difference is maintained on all subsequent 17-trial blocks.

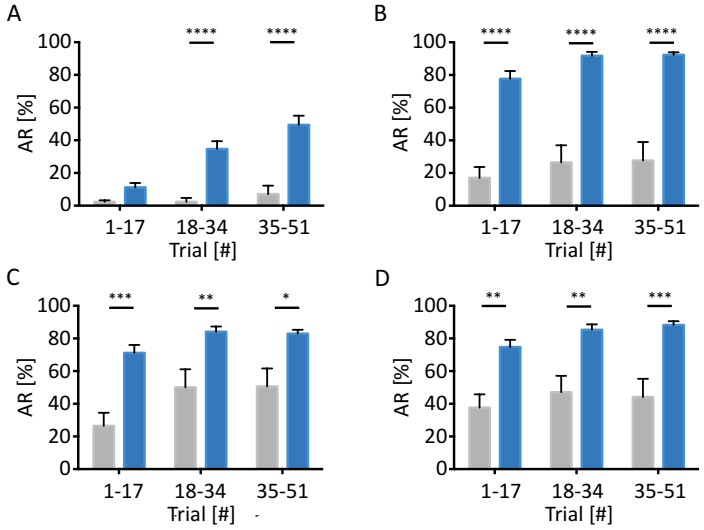

Figure 3.6: The effects on learning appear early and are long lasting. AR rate (%) on ***A*** day 1, ***B*** day 2, ***A*** day 3, and ***B*** day 4. For each day, the trials were divided into three blocks of 17 trials. The AR rate was calculated for each block separately. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test.

Next, we compared how likely the animals are to perform an avoidance response (AR) on trials with light, as compared to trials without light (figure 3.7).

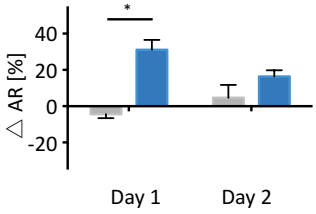

Figure 3.7: Activation of PKCδ+ neurons promotes expression of active avoidance. ΔAR [%] was calculated by substracting the AR rate [%] on trials without light from the AR rate [%] on trials with light. Control group in grey, ChR group in blue. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test.

We find that on the first day of conditioning, the ChR group performed 20% more ARs on trials with light than on trials without light. However, this difference is not evident on day 2. The control group is equally likely to perform an AR on both trials with and without light, from which we conclude that light by itself does not acutely influence active avoidance responding in the 2wAA task.

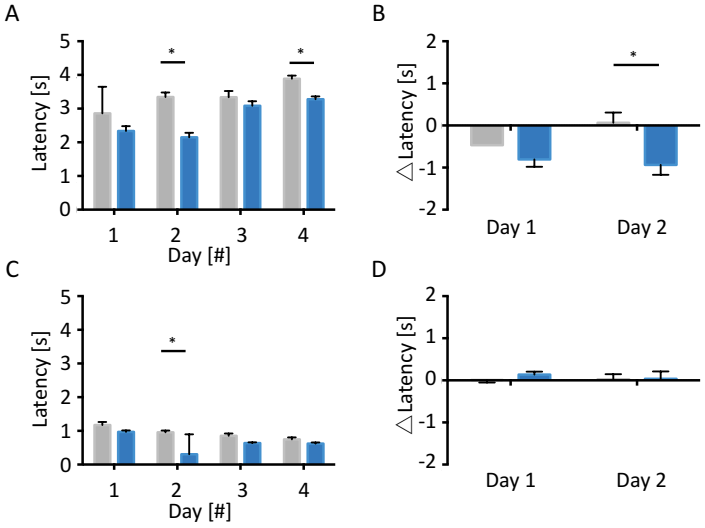

Figure 3.8: Activation of PKCδ$^+$ neurons affects the latency on both ***A*** AR and, ***C*** ER+ trials. The latency to perform an AR is also acutely shorter on trials with light than on trials without light (***B***), but this is not acutely evident on ***D*** ER+ trials. Control group in grey, ChR group in blue.Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test.

Interestingly, the latency with which the mice perform an AR is lower in the experimental group than in the ChR group (figure 3.8 A). At least some of this impact is carried by an acute effect of light on the speed of responding (figure 3.8 B).

Further, we were interested to find how the speed of movement is altered by the optogenetic manipulation. We calculated average animal speed of motion in bins of 1sec for the entire duration of the experiment. We used this data to calculate average speed of motion during the 51 trials. It seems that mice from the ChR group move with a higher average speed during the CS than control group subjects do, especially on the first two days of conditioning (figure 3.9 A and B). Interestingly, here we observe a zig-zag pattern indicating higher speeds on odd-numbered trials than on even-numbered trials. These differences are not obvious on the last two training days (figure 3.9 C and D).

Because of the observed zig-zag pattern of average CS- speed in the ChR group, we explored whether light acutely affects the CS- speed.

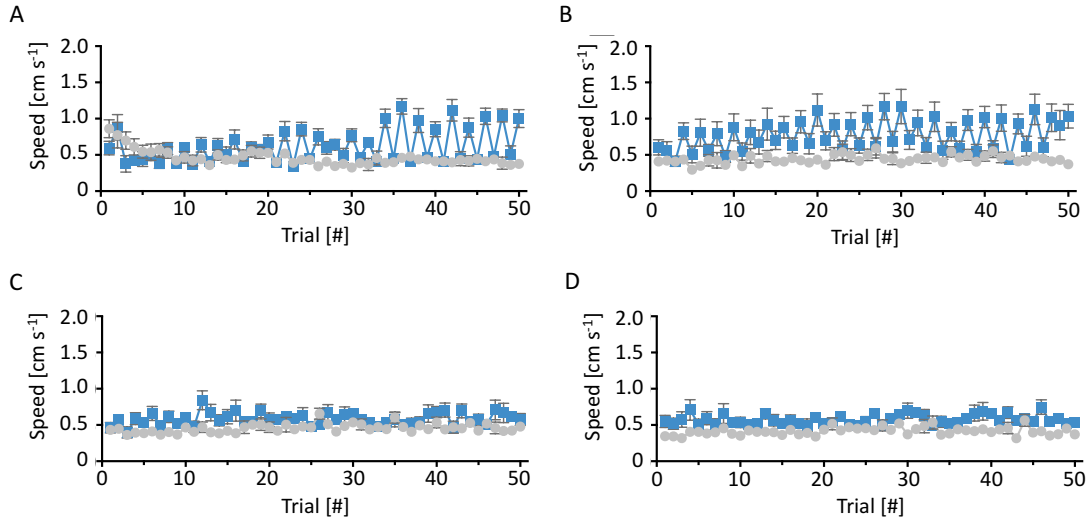

Figure 3.9: Activation of PKCδ$^+$ neurons does affect average speed during CS. Average speed for single trials during CS on ***A*** day 1, ***B*** day 2, ***C*** day 3, and ***D*** day 4. Control group is shown in grey, ChR group is in blue.

It is important to note here that although no light was ever applied on days three and four of training, we still calculated the difference in CS-speed between even numbered trials (with light on day one and two) and odd numbered trials (no light on day one and two). We do not see an acute effect of light on CS-speed in the control group, as the change in speed (Δspeed) stays around zero throughout the conditioning. In the control group, we observed a strong, significant increase of CS-speed both on AR and ER+ trials (figure 3.10).

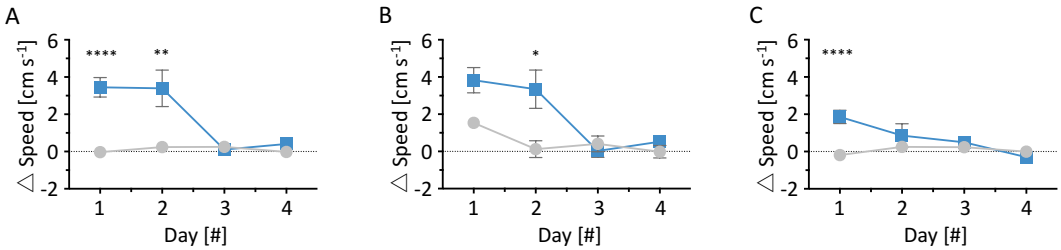

Figure 3.10: Activation of PKCδ$^+$ neurons acutely increases average speed of motion during CS specifically on light+ trials. We calculated difference in speed between even-numbered trials (light+ trials of day 1 and 2) and on odd-numbered trials (light- trials of day 1 and 2). No light was applied on days 3 and four, but the differences were calculated as a control. We performed this calculation for: ***A*** all trials ***B*** for AR trials only, and ***C*** for ER+ trials only. Control group is shown in grey, ChR group is in blue. Significance tests: multiple unpaired paired t-tests, with Holm-Sidak correction for multiple comparisons.

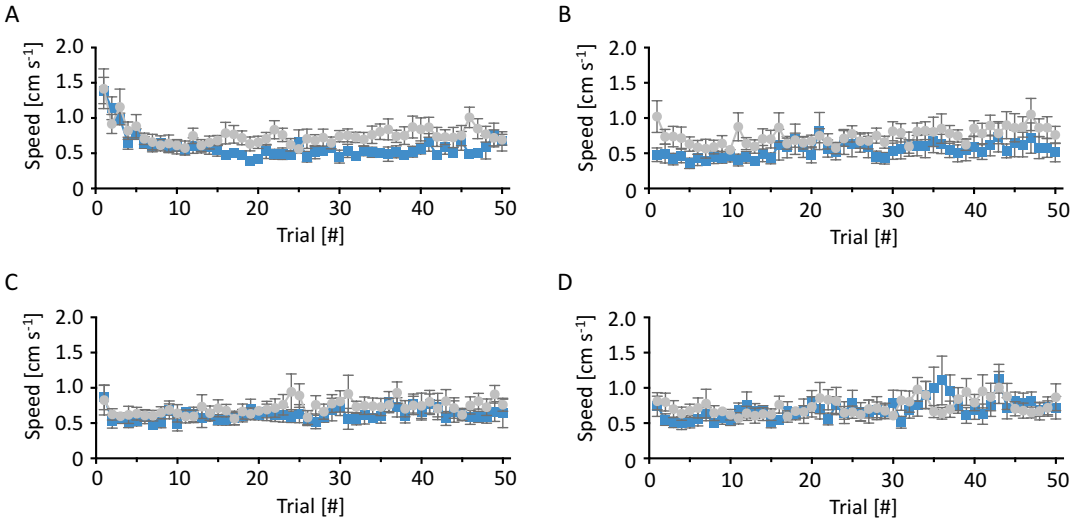

Figure 3.11: Activation of PKCδ$^+$ neurons does not significantly affect average speed during ITI. Average speed for single trials during ITI on ***A*** day 1, ***B*** day 2, ***C*** day 3, and ***D*** day 4. Control group is shown in grey, ChR group is in blue. Significance tests: multiple unpaired paired t-tests, with Holm-Sidak correction for multiple comparisons.

As the light manipulation of PKCδ$^+$ neurons had an acute effect on immobility after the offset of light manipulations during the habituation session, we also explored animal speed during the inter-trial intervals. The slight differences, if any, were not significant neither when comparing each single inter-trial intervals (Figure 3.11), nor when comparing average speeds during all ITIs (Figure 3.12 A). Futhermore, there was no difference in ITI speed following trials with and without light (Figure 3.12 B).

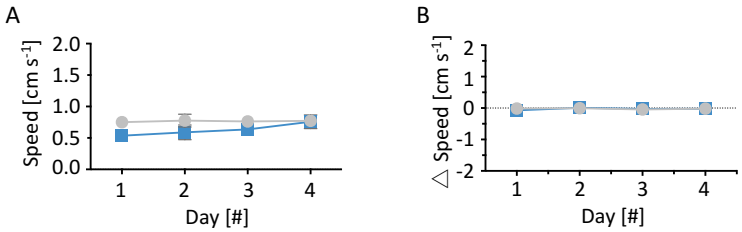

Figure 3.12: Activation of PKCδ$^+$ neurons does not affect average speed during ITI. ***A*** Average speed during ITI on training days 1 to 4. ***B*** Average speed on ITI following even # trials (=light+ trials on day 1 and 2) minus ITI following uneven # trials (=light- trials on day 1 and 2). No light was applied on days 3 and four, but the differences were calculated as a control. Control group is shown in grey, ChR group is in yellow. Significance tests: multiple unpaired paired t-tests, with Holm-Sidak correction for multiple comparisons.

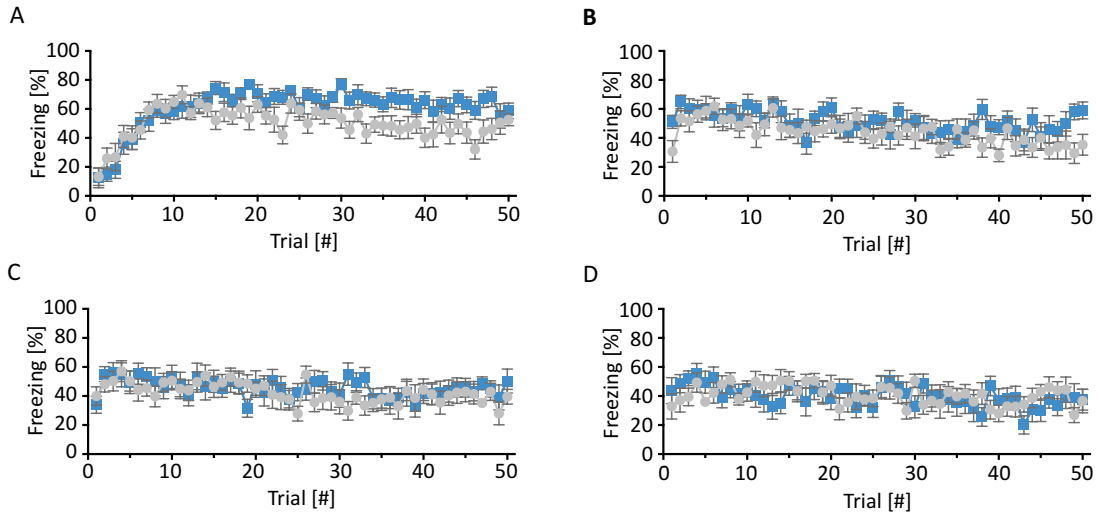

Figure 3.13: Activation of CEA PKCδ+ does not affect post-trial freezing. Difference ***A*** average freezing over all ITI. ***B*** average difference of AR trials with and without light. Control group is shown in grey, ChR group in blue. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test, and multiple unpaired paired t-tests, with Holm-Sidak correction for multiple comparisons.

The striking induction of rebound freezing during the habituation day motivated us to explore freezing during the 2wAA training. Due to the very short CS-only period (max. 5sec) and the specifications used to define freezing (immobility with a minimum duration of 2sec), we focused our analysis efforts to the ITI period. Similarly to the ITI speed, we do not find significant differences between average times spent freezing on single ITI (Figure 3.13).

We decided to explore the freezing on day one in a bit more detail, due to the slight difference evident in both ITI-freezing and ITI-speed during the second half of the training. First we assessed average time spent freezing during all ITIs, and then calculated average freezing during the last 20 ITI. We found a significantly higher amount of freezing in the ChR group as compared to the control group on the last 20 ITIs of conditioning day 1. (Figure 3.14 B) Additionally, we divided each of the last 20 ITI into four blocks of 11sec, then calculated average freezing on each of the blocks. We found that the largest difference is during the first two blocks, and thus in the first 22.5sec of the ITI (Figure 3.14 C).

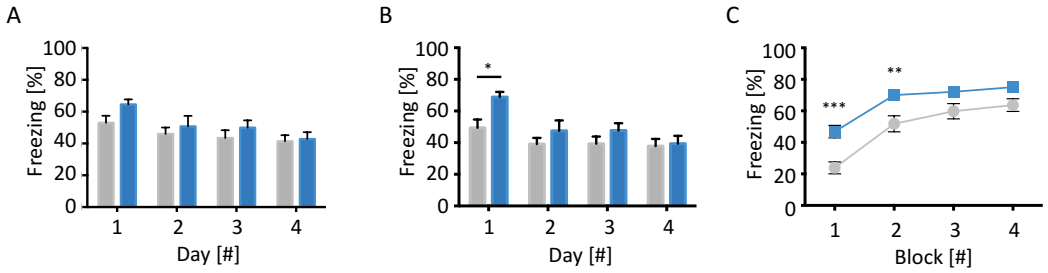

Figure 3.14: Activation of CEA PKCδ$^+$ neurons enhances ITI freezing. ***A*** Average time spend freezing during the ITI on all four days, ***B*** Average time spent freezing during the ITI following last 20 trials, ***C*** ITIs on day 1 from ***B*** were additionally divided into four blocks of 11 sec each. Control group is shown in grey, ChR group in blue. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test.

We also calculated the difference in time spent freezing following AR trials with and AR trials without light (Figure 3.15 A), but found no significant acute effect of light. The same was true when we compared freezing following ER+ trials with and ER+ trials without light (Figure 3.15 B).

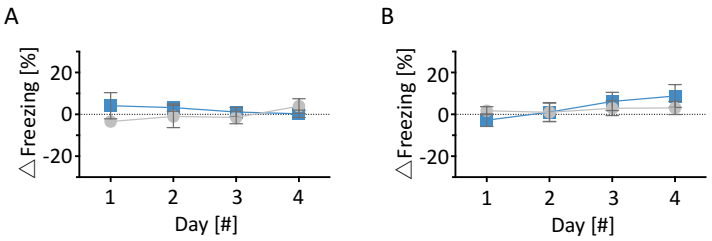

Figure 3.15: Difference in time spent freezing during ITI following trials of type ***A*** AR or ***B*** ER+. Difference is calculated as freezing on ITI following even # trials (=light+ trials on day 1 and 2) minus ITI following uneven # trials (=light- trials on day 1 and 2). Control group is shown in grey, ChR group is in blue. Statistical tests: multiple unpaired paired t-tests, with Holm-Sidak correction for multiple comparisons.

3.2 Optogenetic inhibition of PKCδ$^+$ neurons during 2wAA

We wanted to explore whether the activity of CEA PKCδ$^+$ neurons can bidirectionally influence responding in the 2wAA task. In order to adress this, we used the yellow-light sensitive inhibitory opsin ARCH to inactivate PKCδ$^+$ neurons using the same stimulation pattern described in figure 3.2 C. PKCδ-Cre transgenic mice were injected either with AAV DIO ARCH (ARCH group) or with AAV DIO EGFP (control group)

and implanted with optical fibers. We selected animals based on transgene expression restricted to the CEA and correct placement of optic fibres. These were the only selection criteria applied and resulted in group size of N=10 for the control cohort, and N=11 for the ARCH cohort.

We found no baseline effects of ARCH-based inhibition of CEA PKCδ$^+$ neurons on animals' freezing, neither during yellow light, nor after it (figure 3.16).

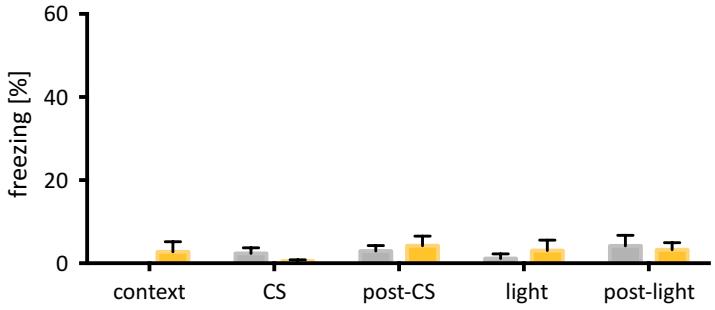

Figure 3.16: There are no baseline effects of ARCH-based inhibition of PKCδ$^+$ neurons on freezing. Control group is shown in grey, ARCH group is in yellow. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test.

Approximately twenty four hours after habituation, the mice were subjected to the first day of 2wAA training. Also here, the light was applied on even-numbered trials only. We find no significant effects on learning: neither the average AR, ER+ nor on the ER- rates (figure 3.17) are different between the ARCH and control groups. We also split all trials into blocks of 17 trials each, but this did not reveal any statistically significant differences between the two cohorts (figure 3.18).

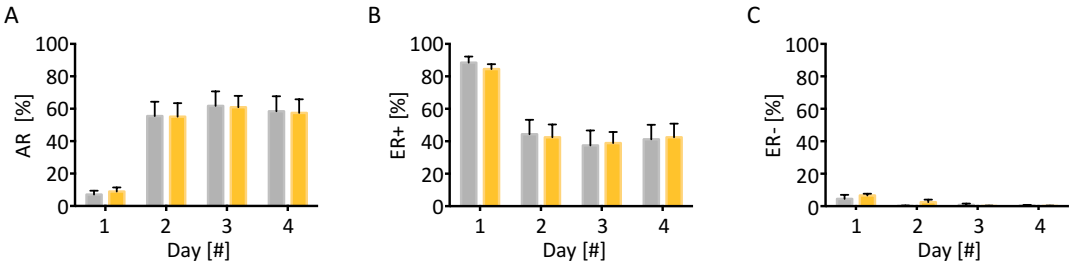

Figure 3.17: Effects of ARCH-based inhibition of PKCδ$^+$ neurons on active responding. Control group is shown in grey, ARCH group is in yellow. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test.

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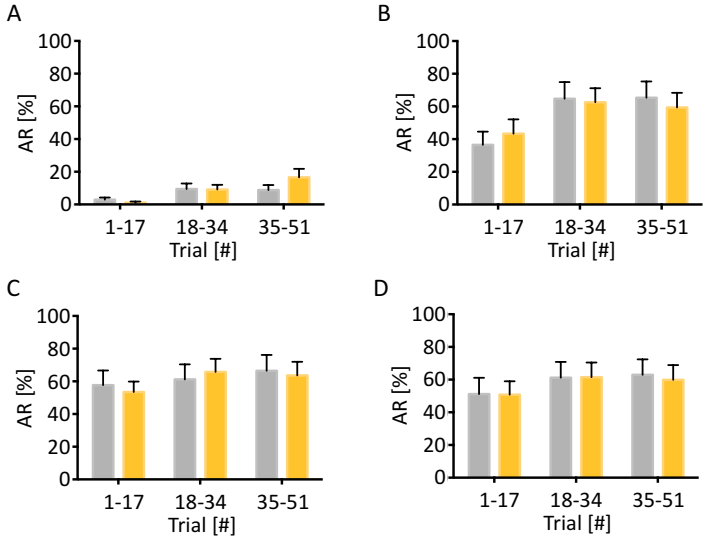

Figure 3.18: Effects of ARCH-based inhibition of PKCδ$^+$ neurons on active responding. ***A*** day 1,. ***B*** day 2, ***C*** day 3, and ***D*** day 4 of 2wAA conditioning. For each day, the trials were divided into three blocks of 17 trials. The AR rate was calculated for each block separately. Control group is shown in grey, ARCH group is in yellow. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test.

Next, we compared how likely the animals are to perform an avoidance response (AR) on trials with light, as compared to trials without light, but find no significant differences (Figure 3.19 A). We also found no significant effect of ARCH-based inhibition on latency to avoid on AR trials and also no difference on latency to escape on ER+ trials (Figure 3.19 B and C).

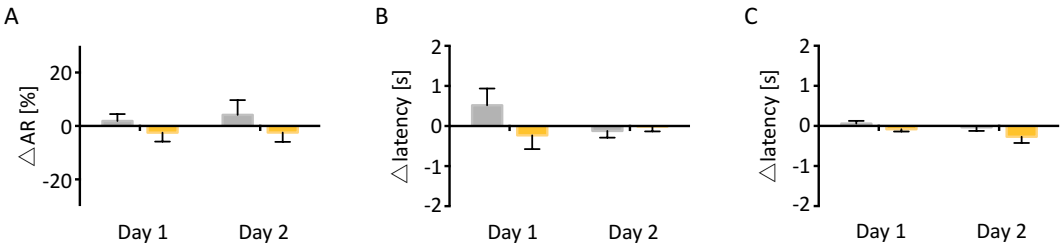

Figure 3.19: Effects of ARCH-based inhibition of PKCδ$^+$ neurons on active responding. ***A*** Difference in AR rates on trials with, vs trials without light,. ***B*** latency difference on AR trials with and without light, ***C*** latency difference on ER+ trials with and without light. Control group is shown in grey, ARCH group is in yellow. Significance tests: multiple unpaired paired t-tests, with Holm-Sidak correction for multiple comparissons.

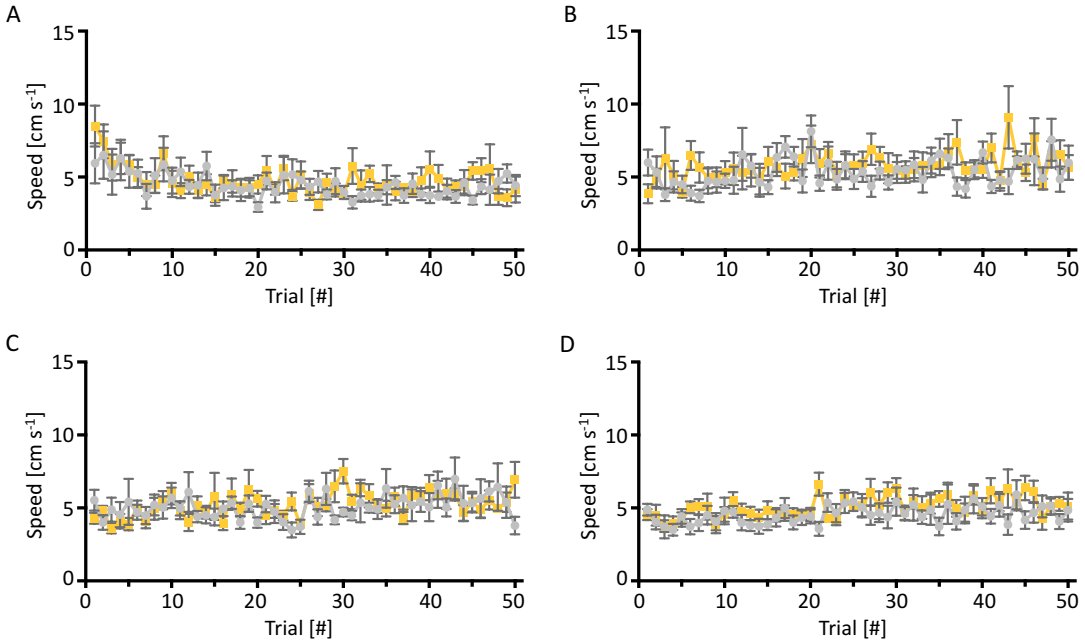

Figure 3.20: Inhibition of PKCδ$^+$ neurons does not affect average speed during CS. Average speed for single trials during CS on ***A*** day 1, ***B*** day 2, ***C*** day 3, and ***D*** day 4. Control group is shown in grey, ARCH group is in yellow. Significance tests: multiple unpaired paired t-tests, with Holm-Sidak correction for multiple comparisons.

Here we also explored how the average speed of movement during CS is changed by the optogenetic inhibition of PKCδ$^+$ neurons but found no significant differences. (Figure 3.20). We also do not find an acute effect of yellow light on average CS- speed, neither when focusing on AR nor when focusing on ER+ trials (Figure 3.21).

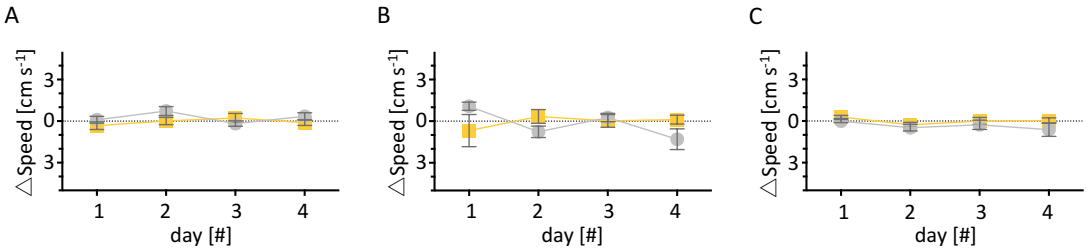

Figure 3.21: Inhibition of CEA PKCδ$^+$ neurons does not acutely affect average speed during CS. ***A*** Difference in average CS-speed during even trials (=light+ trials on day 1+2), vs uneven trials (light- trials on day 1+2) ***B*** Difference in average CS-speed during even# AR-type trials (=light+ trials on day 1+2), vs uneven #AR-type trials (=light- trials on day 1+2) ***C*** Same difference, but for ER+ type trials. Control group is shown in grey, ARCH group is in yellow. Significance tests: repeated measures two-way ANOVA with post-hoc Sidak test.

An assessment of average speeds during ITIs yielded no significant differences between the ARCH and control groups on any of the four conditioning day. Interestingly however, we did observe a trend towards higher speeds, accompanied by an increased variance (Figure 3.22).

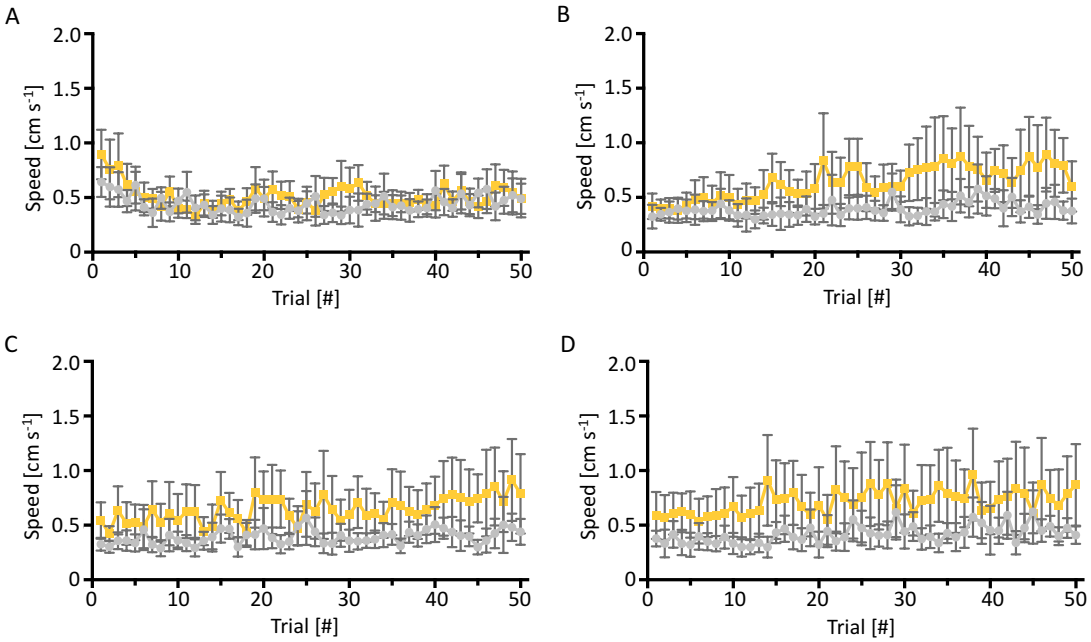

Figure 3.22: Average speed during single ITIs is not affected by ARCH-based inhibition of PKCδ$^+$ neurons, neither on ***A*** day 1, ***B*** day 2, ***C*** day 3, nor ***D*** day 4. Control group is shown in grey, ARCH group is in yellow. Significance tests: repeated measures two-way ANOVA with post-hoc Sidak test.

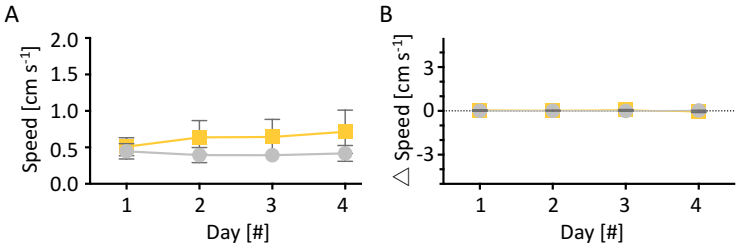

Figure 3.23: Inhibition of CEA PKCδ$^+$ neurons does not affect average speed of motion during ITI. ***A*** Average speed during ITI on training days 1 to 4. ***B*** Average speed on ITI following even # trials (=light+ trials on day 1 and 2) minus ITI following uneven # trials (= light- on day 1 and 2). Control group is shown in grey, ARCH group is in yellow. Significance tests: multiple unpaired paired t-tests, with Holm-Sidak correction for multiple comparisons.

Further, we analysed average speed of motion during the inter-trial intervals. The slight differences were not significant when comparing average speeds during all ITIs (Figure 3.23 A). There was also no difference in ITI speed following trials with and without light (Figure 3.23 B).

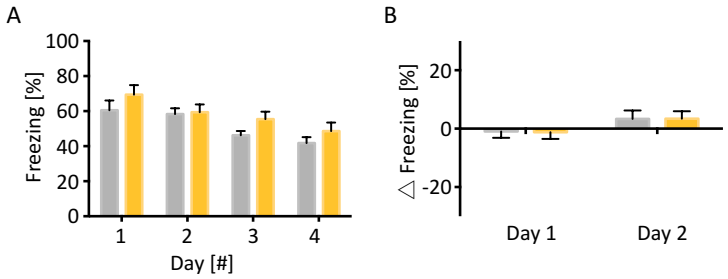

Figure 3.24: Inhibition of PKCδ$^+$ neurons does not affect freezing during ITI. ***A*** Average time (%) spent freezing during ITI. ***B*** Difference in freezing on ITI after trials with and without light. Control group is shown in grey, ARCH group is in yellow. Significance tests: repeated measures two-way ANOVA with post-hoc Sidak test.

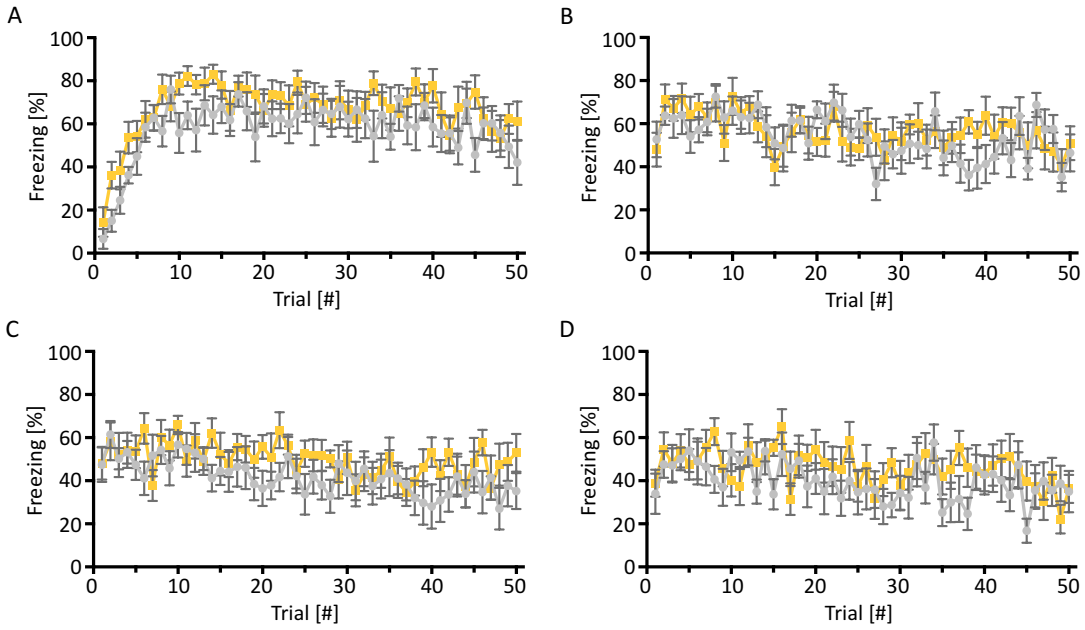

Figure 3.25: Inhibition of PKCδ$^+$ neurons does not affect average freezing during ITI. Average time (%) spent freezing during each ITI displayed separately on ***A*** day 1, ***B*** day 2, ***C*** day 3 and ***D*** day 4. Control group is shown in grey, ARCH group is in yellow. Significance tests: repeated measures two-way ANOVA with post-hoc Sidak test.

We went on to analyse freezing during the ITI, but similarly to the ITI speed, we did not find significant differences between average times spent freezing (figure 3.24 A). Also, no acute effect of light during the trial immediately preceding the assessed ITI was observed (figure 3.24 B).

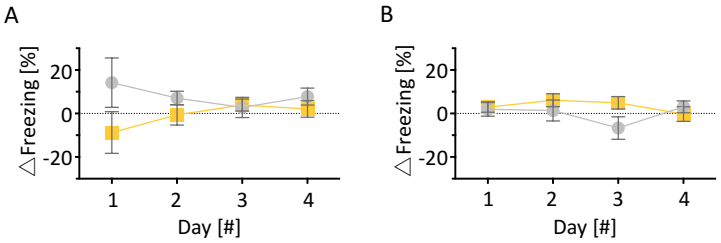

Figure 3.26: Difference in time spent freezing during ITI following trials of type ***A*** AR or ***B*** ER+. Difference is calculated as freezing on ITI following even # trials of the indicated type (=light+ trials on day 1 and 2) minus ITI following uneven # trials of the indicated type (=light- trials on day 1 and 2). Control group is shown in grey, ARCH group is in yellow. Significance tests: multiple unpaired paired t-tests, with Holm-Sidak correction for multiple comparisons.

We again looked at freezing from each ITI event separately, but could not find significant differences on any of the four conditioning days (figure 3.25). We also looked at acute effects of ARCH based inhibition of PKCδ$^+$ neurons on freezing by comparing ITI freezing following AR trials with light and and AR trials without light (figure 3.26 A). We found no significant difference. We did the same for ITI following ER+ trials with/without light (figure 3.26 B) and also there we found no significant differences.

In summary, ARCH based inhibition of CEA PKCδ$^+$ neurons does not affect active avoidance responding of experimental subjects in a 2wAA task. Furthermore, this manipulation also does not affect animal motion as measured by both freezing and by motion speed.

3.3 Optogenetic activation of SOM$^+$ neurons during 2wAA

As described in the introduction, the SOM$^+$ neurons are a prominent population of neurons in the CEA that is largely non-overlapping with the population of PKCδ$^+$ neurons. As they also have known functions in unconditioned and conditioned passive defence, we wondered if and how they can influence animal's active defensive behaviour. In order to assess this, we injected SOM- Cre transgenic mice either with AAV DIO

ChR2 (ChR group) or with AAV DIO EGFP (control group) and implanted them with optical fibers. As always, we trained all animals in the 2wAA task described above and applied our selection criteria (CEA restricted transgene expression and correct fibre placement) afterwards in order to select animals for the analysis. This resulted in group size of N=11 for the control group and N=10 for the ChR group.
First we assessed how optogenetic activation of SOM$^+$ neurons affects locomotion of the experimental subjects, but did not find a significant effect (figure 3.27).

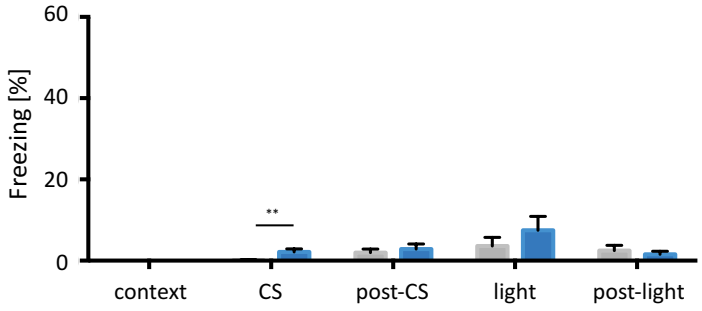

Figure 3.27: ChR-based activation of SOM$^+$ neurons does not influence freezing neither during the blue light, nor after it. Control group is shown in grey, ChR group is in blue. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test.

A day after the habituation session, mice were subjected to the first day of 2wAA training on which blue light was applied on even-numbered trials only. We assessed how the ChR-based activation of CEA SOM$^+$ neurons affects performance of active avoidance. We find no effects on learning of 2wAA (Figure 3.28, and figure 3.29).

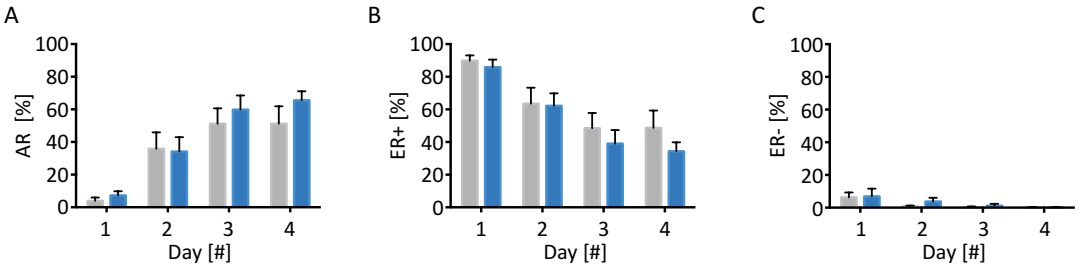

Figure 3.28: Central amygdala SOM$^+$ neurons do not affect learning of active avoidance. Activation of SOM$^+$ ***A*** does not affect AR, ***B*** ER+, nor ***C*** ER- rates. Values indicate % of total trials). Control group is shown in grey, ChR group is in blue. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test

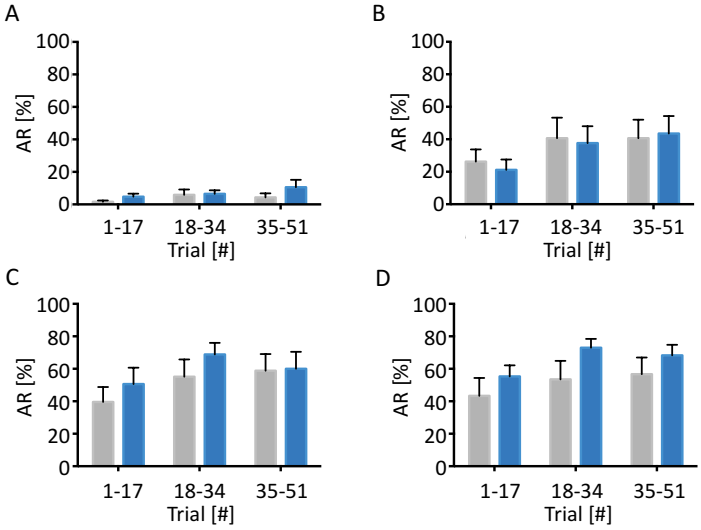

Figure 3.29: For each day, the trials were divided into three blocks of 17 trials. The AR rate was calculated for each block separately and is shown for: ***A*** day 1, ***B*** day 2, ***A*** day 3, and ***B*** day 4. Control group is shown in grey, ChR group is in blue. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test

We tested how likely the mice are to perform an avoidance response (AR) on trials with light, as compared to non-manipulated trials. We found a small, but significant effect. When compared to the EGFP control group, we found the ChR group less likely to perform AR on trials with light than on trials without light (figure 3.30).

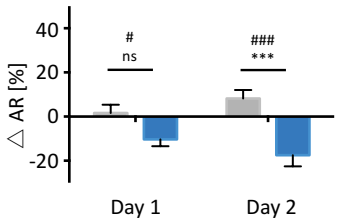

Figure 3.30: Activation of SOM$^+$ neurons inhibits expression of active avoidance. Evident as lower likelihood of AR responses on trials with light than on trials without light. Control group in grey, ChR group in blue. * symbols denote significant p-values from repeated measure two-way ANOVA with post-hoc Sidak test. # symbols stand for significant p-values from multiple t-test comparison with Holm-Sidak correction.

The latency with which the mice perform avoidance responses seems also to be affected. When compared to the control group, the ChR cohort not only performed fewer AR on trials with light, but also performed them slower (figure 3.31 B). The ER responses seem unaffected by light manipulation (figure 3.31 C and D).

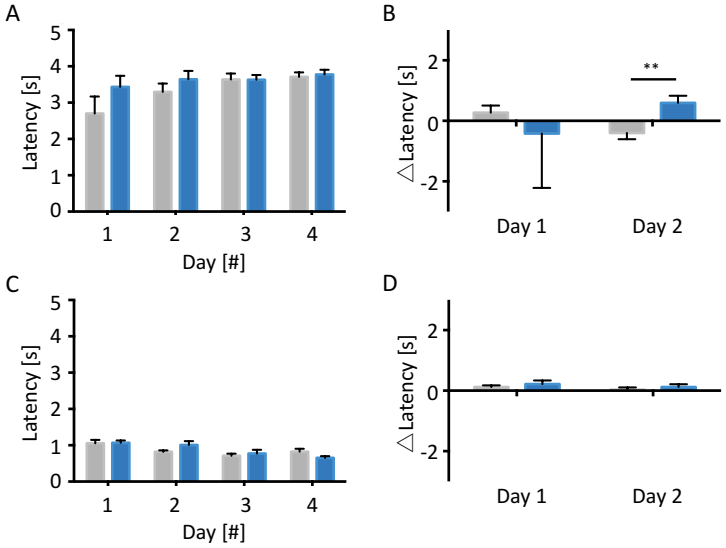

Figure 3.31: Activation of SOM$^+$ neurons does not affect the average latency on neither ***A*** AR nor on, ***C*** ER+ trials. There is however a difference between trials with and without light, specifically on ***B*** AR, nor on ***D*** ER+ trials. Control group in grey, ChR group in blue. Statistical tests: multiple unpaired paired t-tests, with Holm-Sidak correction for multiple comparisons.

We examined how the speed of movement is changed by optogenetic activation of SOM$^+$ neurons. It seems that on a few trials small differences in CS speed might exist (Figure 3.32). We pursued the lead further by comparing differences in CS- speed on trials with and without light in ChR and control groups. We find lower speeds of motion during the CS on trials with light, and this is especially pronounced in AR trials (Figure 3.33). This is in line with the observed higher latency of responding during AR trials with light (Figure 3.31).

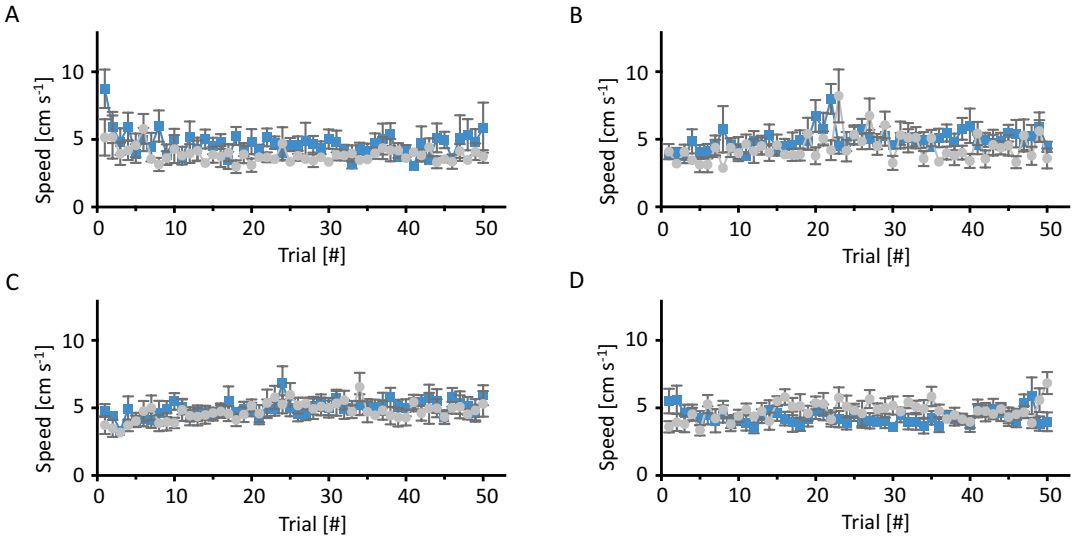

Figure 3.32: Activation of SOM$^+$ neurons might affect animal motion during CS. Average speed for single trials shown during CS on ***A*** day 1, ***B*** day 2, ***C*** day 3, and ***D*** day 4. Control group is shown in grey, ChR group is in blue.

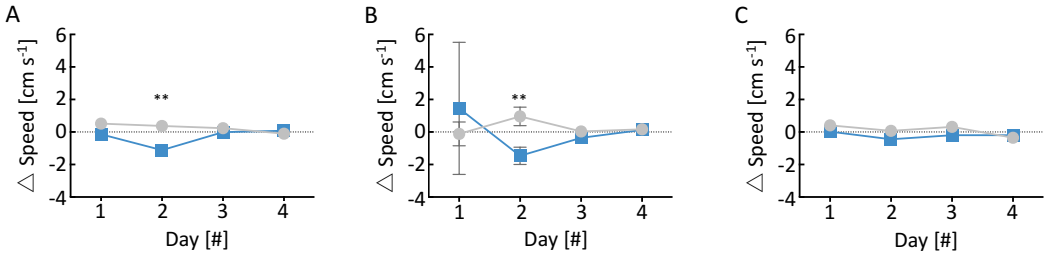

Figure 3.33: Optogenetic ctivation of SOM$^+$ neurons acutely decreases average speed during CS. Difference in CS speed on ***A*** all trials, ***B*** AR trials, and ***C*** ER+ trials. Difference is calculated by subtracting average speed on odd # trials of a given type (=light- trials on day 1 and 2) from average speed on even # trials of a given type (=light- trials on day 3 and 4). Control group is shown in grey, ChR group is in blue. Statistical tests: multiple unpaired paired t-tests, with Holm-Sidak correction for multiple comparisons.

We also examined animal motion during inter-trial intervals by computing ITI speed of motion (Figure 3.34) and ITI freezing (Figure 3.35). Although we did not observe significant differences in speed of motion during the ITI, the freezing analysis revealed an interesting phenotype. In figure 3.35 we observe a zig-zag curve on day two, indicative of an acute effect of activation of SOM$^+$ neurons on the freezing.

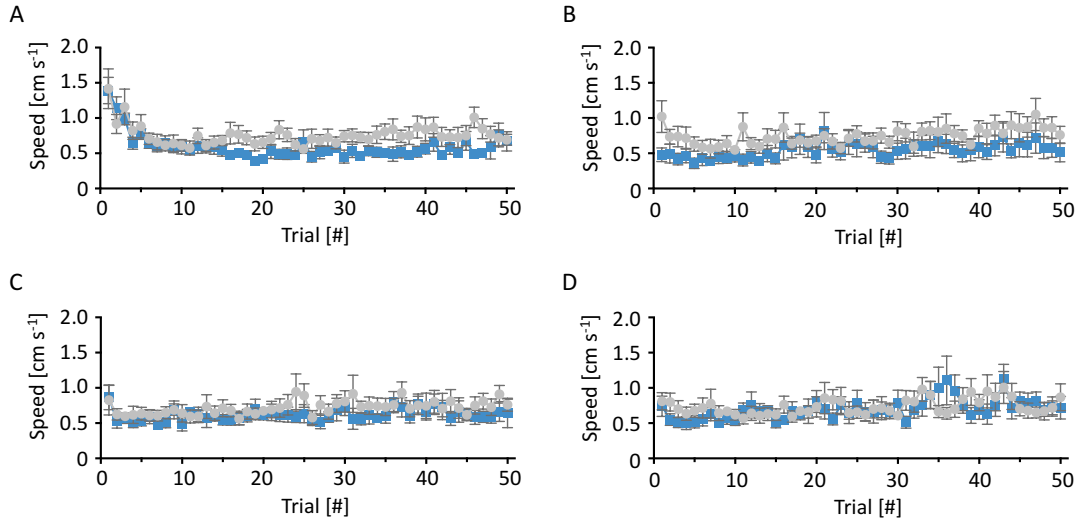

Figure 3.34: Activation of CEA SOM$^+$ neurons seems not to significantly affect speed of motion during ITI. Average time spent freezing during each ITI on ***A*** day 1, ***B*** day 2, ***A*** day 3, and ***B*** day 4 of conditioning. Control group is shown in grey, ChR group in blue. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test

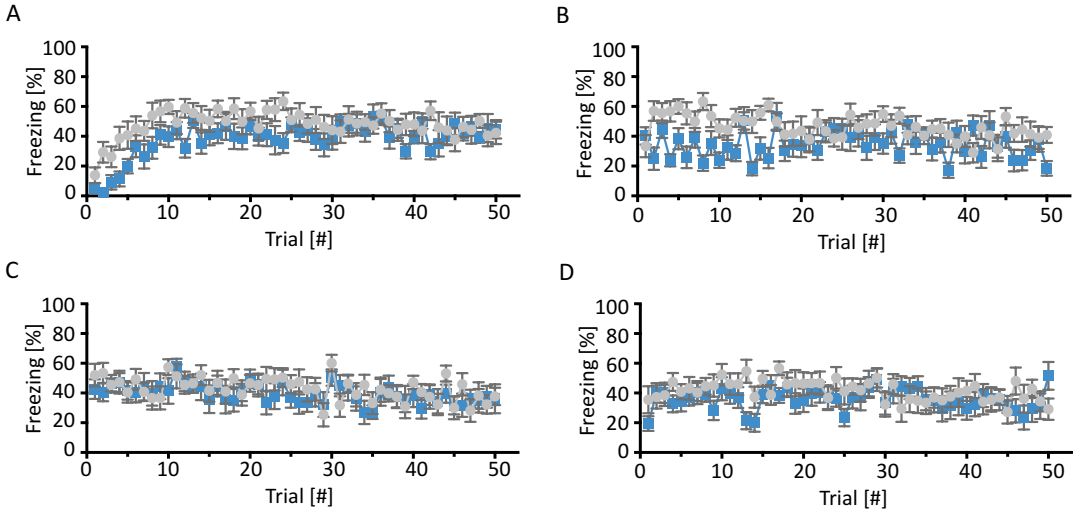

Figure 3.35: Activation of SOM$^+$ neurons seems to affect post-trial freezing. Average time spent freezing during ITI after each trial on ***A*** day 1, ***B*** day 2, ***A*** day 3, and ***B*** day 4 of conditioning. Control group is shown in grey, ChR group is in blue. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test.

In order to follow up on this, we first calculated average ITI freezing, but did not observe a significant difference between ChR and control groups. However, when we calculated changes in ITI freezing following ER+ trials with light and without light, significantly lower freezing was observed in the ChR group on trials without light. This was not the case on inter trial intervals following AR trials (figure 3.36).

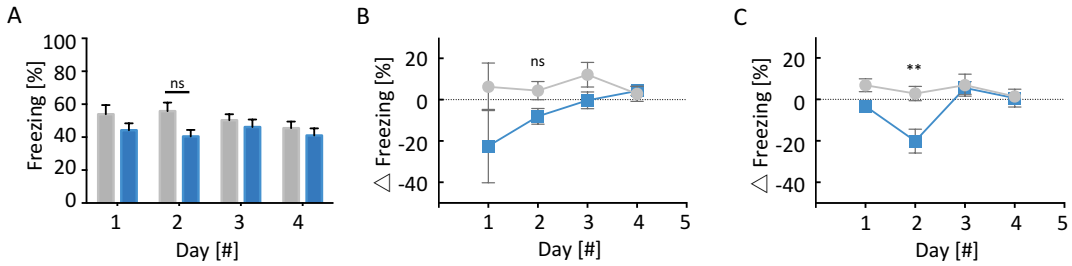

Figure 3.36: Activation of SOM$^+$ neurons affect freezing. ***A*** Average time spent freezing during the ITI on all four days. Difference in freezing due to light on ITI following trials of ***B*** AR and ***C*** ER+ type. Difference is calculated by subtracting average freezing on ITI following odd # trials of a given type (=light- trials on day 1 and 2) from average freezing on ITI following even # trials of a given type (=light- trials on day 3 and 4). Control group is shown in grey, ChR group in blue. Statistical tests: multiple unpaired paired t-tests, with Holm-Sidak correction for multiple comparisons.

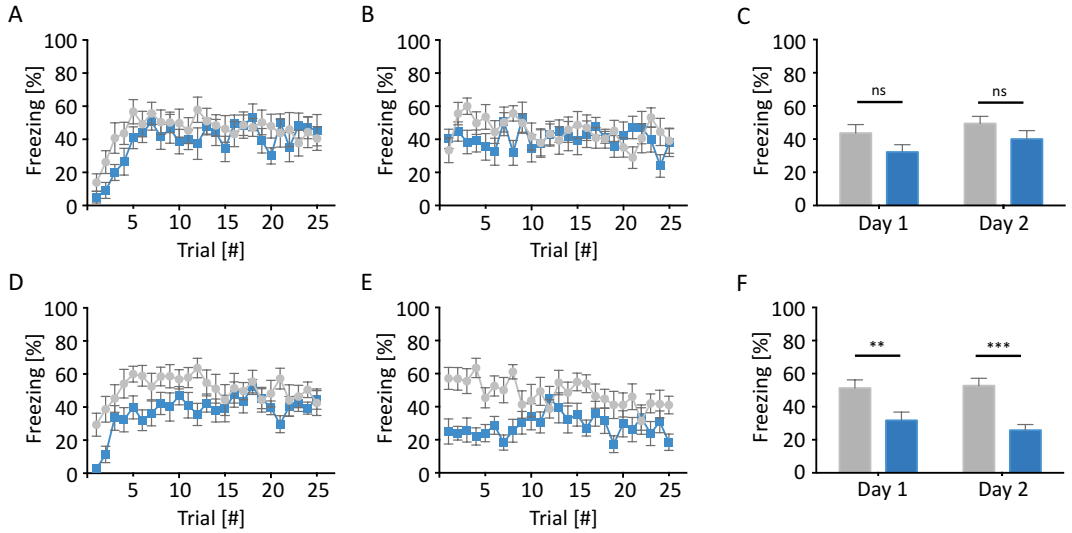

Figure 3.37: Influence of SOM$^+$ neuron activation on ITI freezing is evident especially on early trials. Average time spent freezing during ITI following no-light trials on ***A*** day 1 and ***B*** day 2. First ten trials shown in ***A*** and ***B*** were averaged and are shown in ***C***. The same calculation, but for ITI following trials with light. Control group is shown in grey, ChR group is in blue. Statistical tests for C and F: repeated measures two-way ANOVA with post-hoc Sidak test

Additionally we also divided the inter-trial intervals into ITIs following trials with and without light and compared them directly between the control and ChR groups. Freezing following trials without light was not significantly different in the control and ChR groups. However, freezing after trials with light was significantly reduced in the ChR group. The difference was especially pronounced in the first half of the conditioning on each of the two days (figure 3.37 F).

3.4 Optogenetic inhibition of SOM$^+$ neurons during 2wAA

Since the PKCδ$^+$ neurons did not show potential to bidirectionally influence active responding in the 2wAA task, we were wondering if this is also the case for the SOM$^+$ neurons. In order to address this, we used the yellow-light sensitive inhibitory opsin ARCH to inactivate SOM$^+$ neurons using the same stimulation pattern described in figure 3.2 C. SOM- Cre transgenic mice were injected either with AAV DIO ARCH (ARCH group) or with AAV DIO EGFP (control group) and implanted with optical fibres. We selected animals based on transgene expression restricted to the CEA and correct placement of optic fibres. These were the only selection criteria applied and resulted in group size of N=15 for the control cohort, and N=15 for the ARCH cohort.

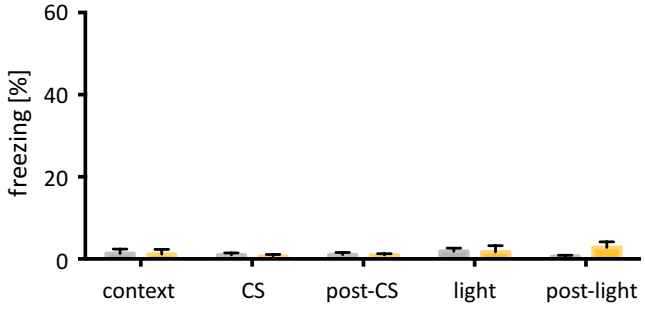

Figure 3.38: Baseline effects of ARCH-based inhibition of SOM$^+$ neurons. Control group is shown in grey, ARCH group is in yellow. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test.

During habituation session, we found no baseline effects of ARCH-based inhibition of CEA SOM$^+$ neurons on animals' freezing, neither during yellow light, nor after it (figure 3.38). A day after habituation, the mice underwent their first 2wAA training, during which the light was applied on even-numbered trials only. We find a significantly in-

creased active responding of the ARCH-group, as evident from elevated AR and lower ER+ rates (figure 3.39 and figure 3.40) are different between the ARCH and control groups.

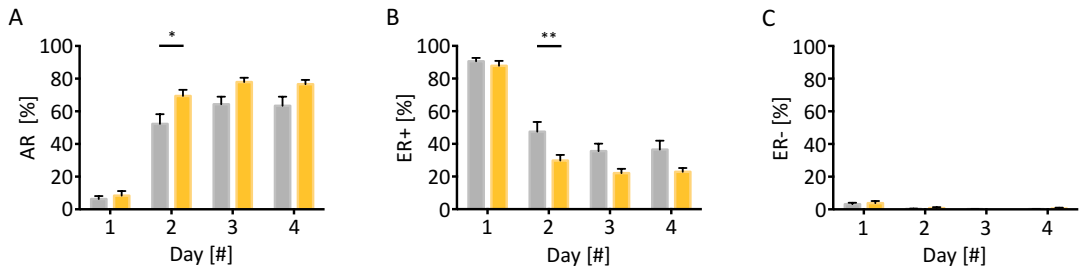

Figure 3.39: Effects of ARCH-based inhibition of SOM$^+$ neurons on active responding. Control group is shown in grey, ARCH group is in yellow. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test.

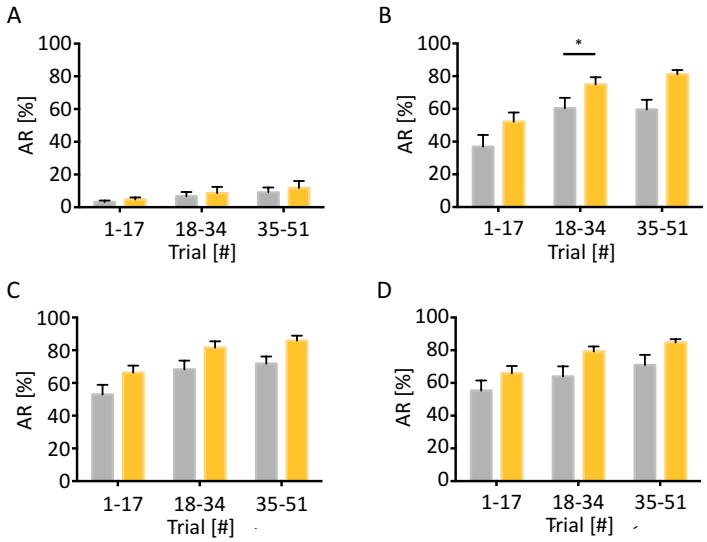

Figure 3.40: Effects of ARCH-based inhibition of SOM$^+$ neurons on active responding. ***A*** day 1,. ***B*** day 2, ***C*** day 3, and ***D*** day 4 of 2wAA conditioning. On each training day, the trials were divided into three blocks of 17 trials each. The AR rate was calculated for each block separately. Control group is shown in grey, ARCH group is in yellow. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test.

Next, we compared how likely the animals are to perform an avoidance response (AR) on trials with light, as compared to trials without light, but find no significant differences (figure 3.41 A). We also found no significant effect of ARCH-based inhibition on latency to avoid on AR trials and also no difference on latency to escape on ER+ trials

(figure 3.42 B and C).

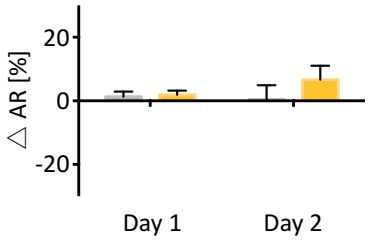

Figure 3.41: No accute effects of ARCH-based inhibition of SOM$^+$ neurons on active avoidance. The difference in AR was calculated by subtracting AR rate on trials without light form the AR rate on trials with light. Control group is shown in grey, ARCH group is in yellow. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test

Next, we also explored the effect of CEA SOM$^+$ neuron inhibition on the speed of motion during the conditioned stimuli (figure 3.43), but found no obvious differences. We also do not find an acute effect of yellow light on average CS-speed, neither when focusing on AR only, nor when specifically looking at ER+ trials (figure 3.44 B and C).

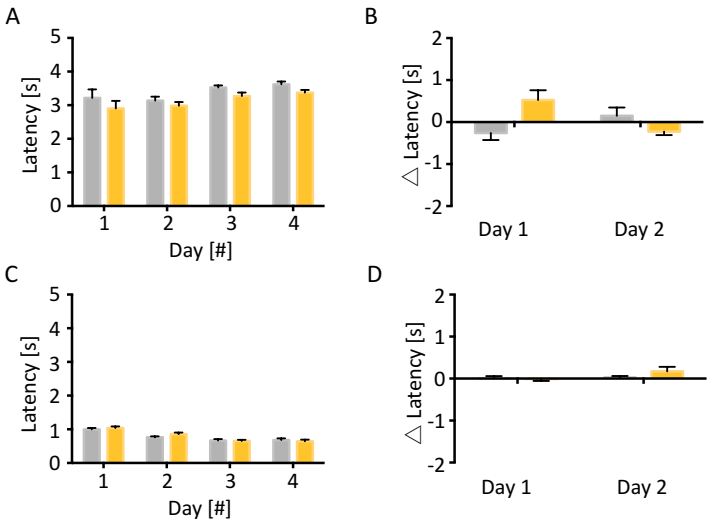

Figure 3.42: Inhibition of SOM$^+$ neurons does not affect the latency on either ***A*** AR nor on, ***C*** ER+ trials. There is also no significant difference between trials with and without light, neither during ***B*** AR trials, nor during ***D*** ER+ trials. Control group in grey, ARCH group in yellow. Statistical tests: multiple unpaired t-tests, with Holm-Sidak correction for multiple comparisons.

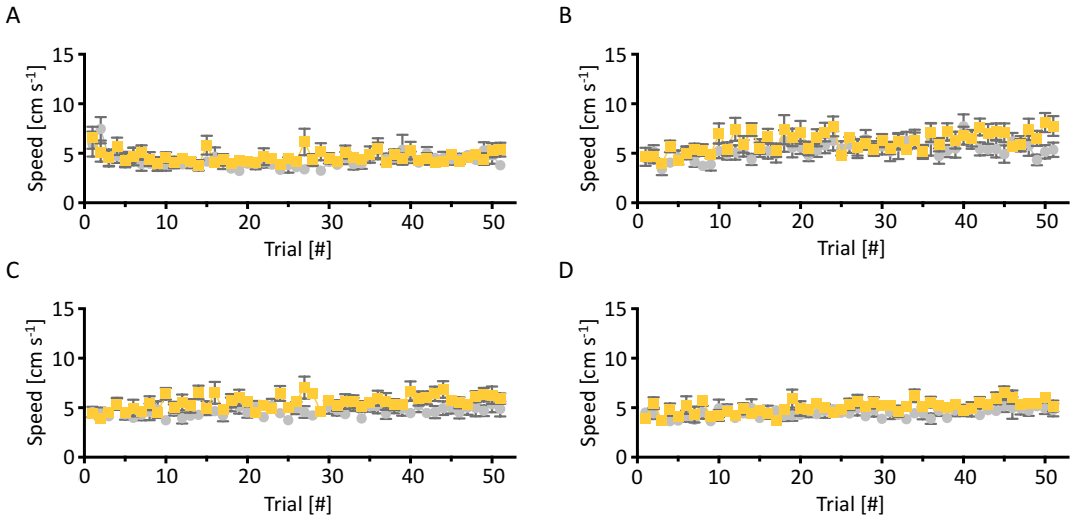

Figure 3.43: Inhibition of SOM$^+$ neurons does not affect average speed during CS. Average speed for single trials during CS on ***A*** day 1, ***B*** day 2, ***C*** day 3, and ***D*** day 4. Control group is shown in grey, ARCH group is in yellow. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test.

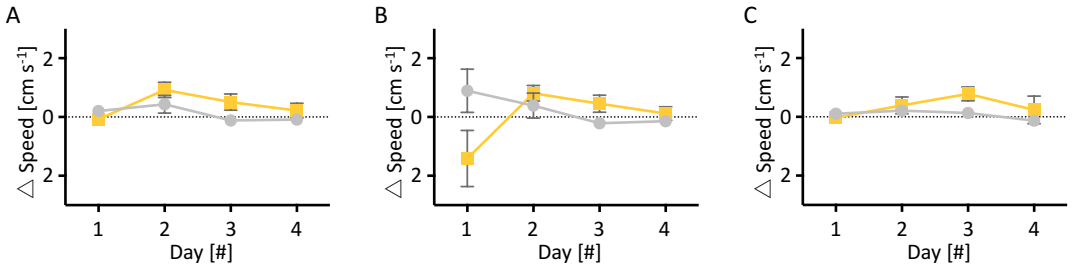

Figure 3.44: Inhibition of SOM$^+$ neurons does not affect average speed during CS on trials with light, compared to trials without light. Differences in average speed during CS on ***A*** all trials, ***B*** AR trials, and ***C*** ER+ trials. Differences were calculated by subtracting average speed on odd numbered trials of a given type (=light- trials on day 1 and 2) from speed on even numbered trials of a given type (=light+ trials on day 1 and 2). Control group is shown in grey, ARCH group is in yellow. Statistical tests: multiple unpaired t-tests, with Holm-Sidak correction for multiple comparisons.

An assessment of average speeds during ITIs also showed no significant differences between the ARCH and control groups on any of the four conditioning days (figure 3.45 and 3.46 A). Furthermore, when we compared the average speed during ITI following a trial with light with speed during ITI following a trials without light, we found no differences neither within, nor across groups (figure 3.46 B).

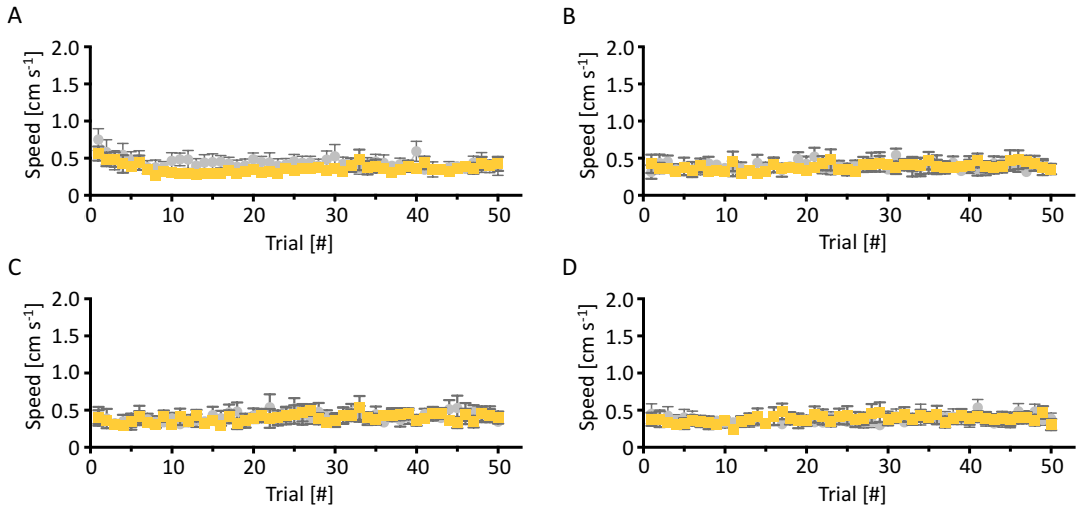

Figure 3.45: Average speed during single inter-trial intervals on ***A*** day 1, ***B*** day 2, ***C*** day 3, and ***D*** day 4. Control group is shown in grey, ARCH group is in yellow. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test.

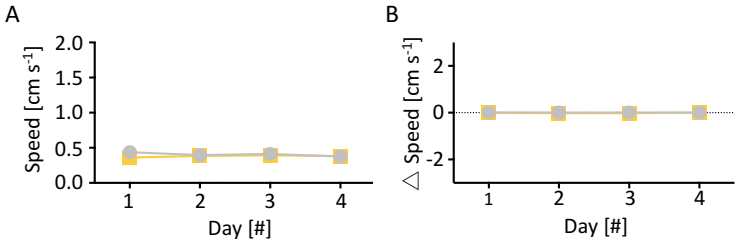

Figure 3.46: Inhibition of SOM$^+$ neurons does not affect average speed during ITI. ***A*** Average speed during ITI on training days 1 to 4. ***B*** Average speed on ITI following even # trials (=light+ trials on day 1 and 2), minus Iaverage speed on TI following uneven # trials (=light- trials on day 1 and 2). Control group is shown in grey, ARCH group is in yellow. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test.

We went on to analyse freezing during the ITI, but similarly to the ITI speed, we did not find significant differences between average times spent freezing (figure 3.47 A). Also, no acute effect of light during the trial immediately preceding the assessed ITI was observed (figure 3.47 B).

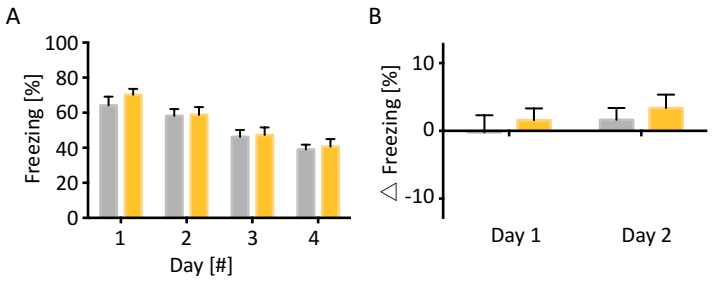

Figure 3.47: Inhibition of SOM$^+$ neurons does not affect average freezing during ITI. ***A*** Average time (%) spent freezing during ITI. ***B*** Difference in freezing on ITI after trials with minus after trials without light. Control group is shown in grey, ARCH group is in yellow. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test

We again looked at freezing from each ITI event separately, but could not find significant differences on any of the four conditioning days (figure 3.48). We also looked at acute effects of ARCH based inhibition of SOM$^+$ neurons on freezing by comparing ITI freezing following AR trials with light and and AR trials without light (Figure 3.49 A), but found no significant differences.

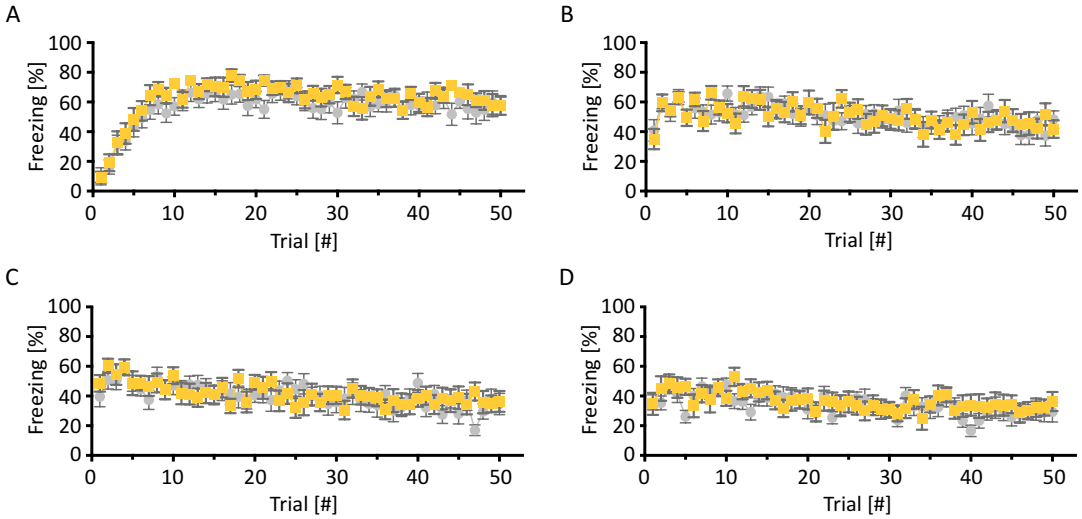

Figure 3.48: Inhibition of SOM$^+$ neurons does not affect average freezing during ITI. Average time (%) spent freezing during each ITI displayed separately on ***A*** day 1, ***B*** day 2, ***C*** day 3 and ***D*** day 4. Control group is shown in grey, ARCH group is in yellow. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test

We did the same for ITI following ER+ trials with and without light (Figure 3.49 B) and also there we found no significant differences.

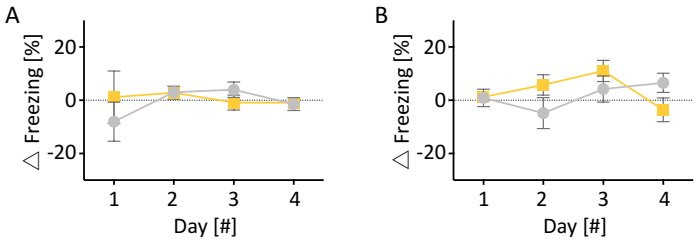

Figure 3.49: Difference in time spent freezing during ITI following trials of type ***A*** AR or ***B*** ER+. Difference is calculated as freezing on ITI following even # trials (=light+ trials on day 1 and 2) minus ITI-freezing following uneven # trials (=light- trials on day 1 and 2). Control group is shown in grey, ARCH group is in yellow. Statistical tests: multiple unpaired t-tests, with Holm-Sidak correction for multiple comparisons.

3.5 Calcium imaging of PKCδ+ neurons during 2wAA

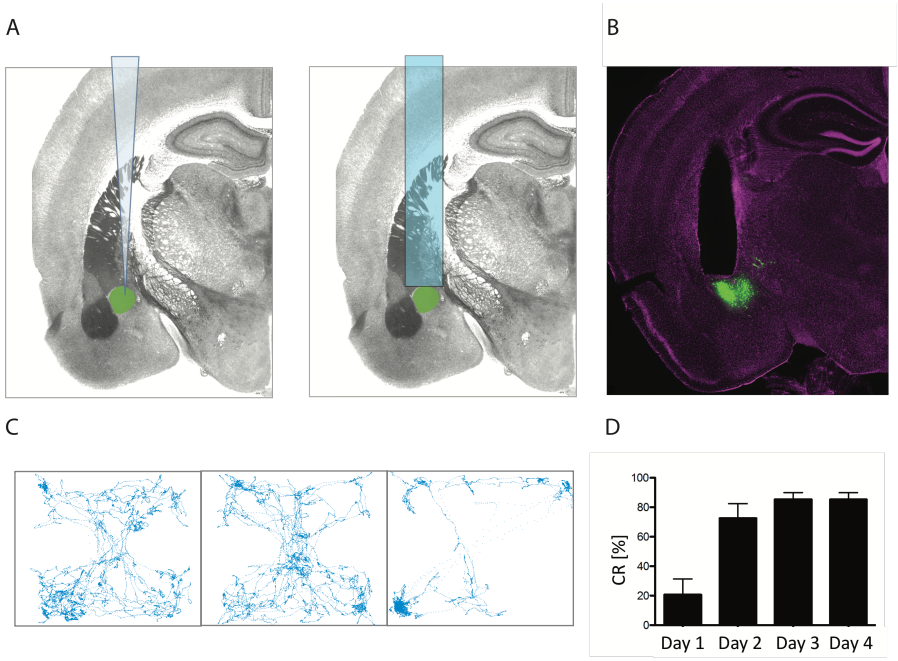

Figure 3.50: Ca2+ imaging of PKCδ+ neurons using microendoscopes. ***A***-left PKCδ-Cre mice were unilaterally injected with AAV DIO GCaMP6s into the CEA. ***A***-right A week later, they were implanted with a GRIN lens at the level of the CEA. ***B*** A brain slice from a sucessfuly imaged animal. GCaMP6s expressing cells are green, DAPI is in magenta. ***C*** Animal movement profiles during 6minutes surrounding the first tone presentation on habituation (left), first day (middle) and second day (right) of 2wAA conditioning. ***D*** The microscope-mounted experimental animals were able to learn the 2wAA task.

The intriguing results from optogentics experiments motivated us to explore the natural activity of CEA, and especially of PKCδ^+ neurons during two-way active avoidance. We were hoping to discover physiological signatures of transitions from active defence to passive defence, as well as clues about why the optogenetic activation of PKCδ^+ neurons boosted learning and expression of active avoidance, whereas ARCH-mediated inhibtion had no effect.

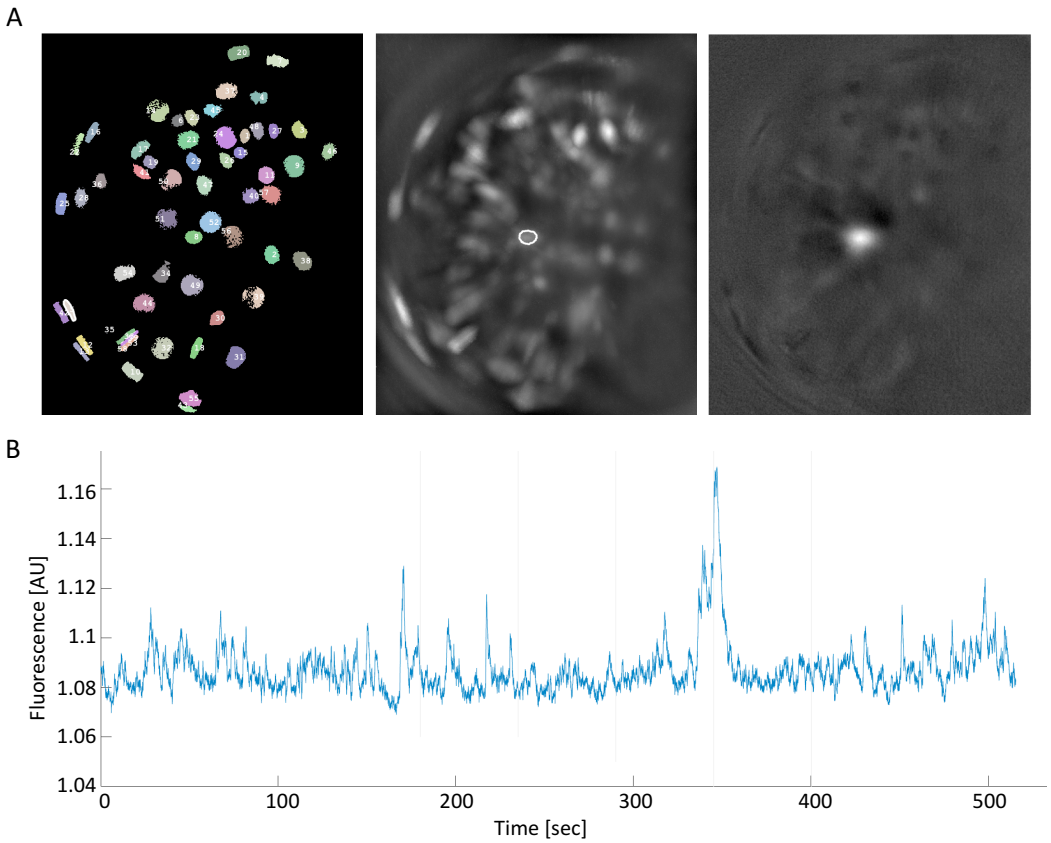

Figure 3.51: Example of a cell taken into further analysis. ***A***-left all automatically extracted cell masks. ***A***-middle maximum intensity projection of the field of view with the selected cell mask. ***A***-right IC unmixing image. ***B*** Full (detrended) raw fluorescence trace.

First we explored the possibilities to record the activity of PKCδ^+ neurons using extracellular activity recordings. However, due to low unit yield per animal which is specific to recordings from the CEA, we also looked into other recording techniques. Calcium imaging using miniature, head-mountable microendoscopes is an alternative that could potentially allow simultaneous recording of dozens of neurons.

We thus injected recombinant AAVs containing a Cre-dependant GCaMP6s construct into the CEA of PKCδ- Cre mice. A week after this injection, we implanted the experimental subjects with a 0.6mm GRIN lens (figure 3.50). After another seven days, we started checking for fluorescence with an nVista microendoscope on a weekly basis until the fluorescent signals were sufficiently strong for imaging.

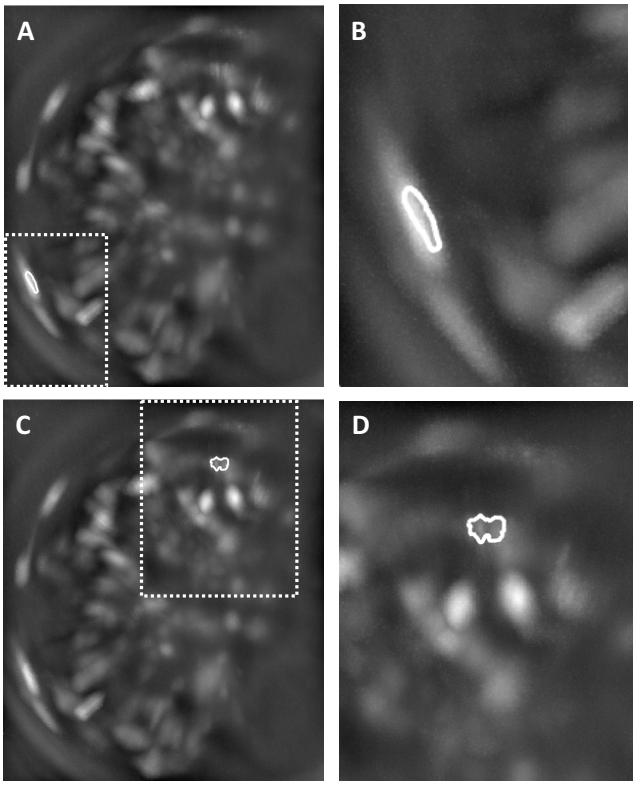

Figure 3.52: Example of a cell masks not taken into further analysis. ***A*** Maximum intensity projection with a superimposed cell mask border (in white). Region marked with the dashed rectangle is magnified and displayed in ***B***. The cell was excluded because the cell shape is elongated and atypical. ***C*** Maximum intensity projection superimposed with another cell mask border (in white). Region marked with the dashed rectangle is magnified and displayed in ***C***. The cell was excluded because of its fuzzy, atypical shape.

At this point, we mounted the experimental subjects with baseplates that allow easy and reliable mounting of the scope onto the animal's head and just above the GRIN lens, always at the same focal plane (for more details, see the Methods section). After another few days of handling, the mice were ready for two-way active avoidance training. We used the same training procedure described previously (see Methods) and found that

the mice with head-mounted microendoscopes were capable of learning the 2wAA task (Figure 3.50). We selected those animals for analysis that had good GCaMP6s signals and whose GRIN lens placement was in the central amygdala.
First we pre-processed the videos by passing them through a FFT-based band pass filter with the purpose of removing noise and broad neuropil signals. After that we performed motion correction and automatic extraction of fluorescent traces using a PCA-ICA based algorithm (for details see Methods and [Mukamel et al., 2009]). We imported those traces into MATLAB and performed all further analysis with custom written scripts. After detrending the traces, we superimposed the automatically extracted cell filters with mean and maximum intensity projections of all frames in order to evaluate whether the cell filters actually correspond to cells. If the cell filters did not correspond to clearly defined single cells, the trace was excluded from further analysis. An example of a cell mask and trace taken into further analysis is shown in figure 3.51. Two example of cells with atypical shapes that were not taken into analysis are shown in 3.52.

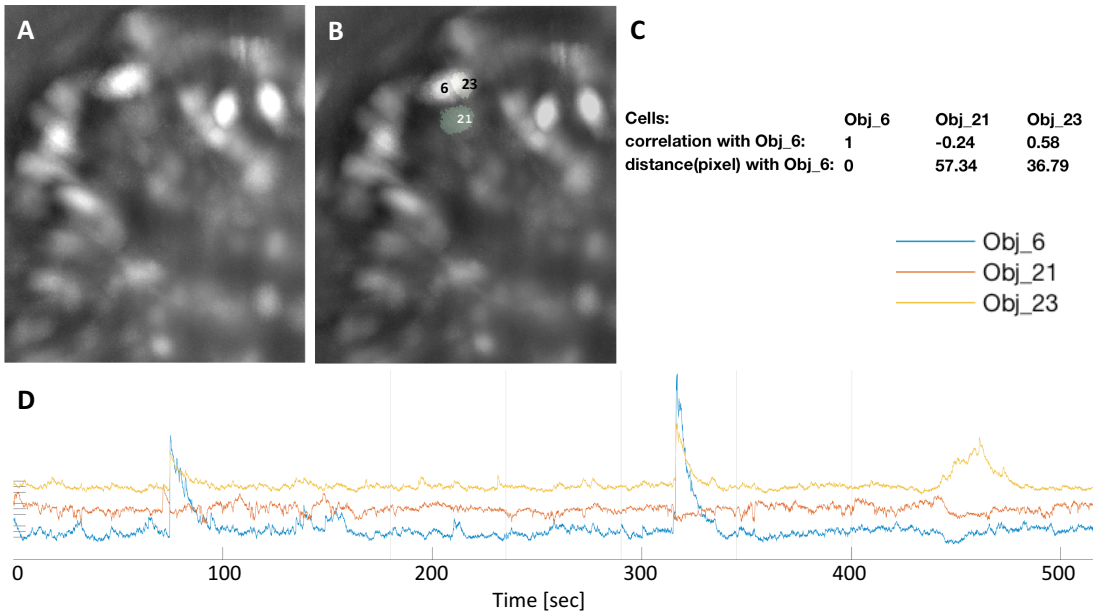

Figure 3.53: Example of neighbouring cells whose traces show high correlation. ***A*** Maximum intensity projection of cells imaged through a GRIN lens using a microendoscope (6 = Obj_6, 23 = Obj_23, 21 = Obj_21). ***B*** Maximum intensity projection superimposed with three neighbouring cell masks. ***C*** Correlation of the cell traces extracted from the three cell masks with the cell mask Obj6. ***D*** Normalised fluorescence traces. Three marks surrounding each trace on the left edge correspond (from top to bottom) to +1%, 0% and −1% change in ΔF/F0.

In order to further maximise the probability that the fluorescent traces correspond to signals from single cells, we performed correlation analysis of all fluorescent traces and evaluated in detail whether traces with high correlation ($r > 0.5$) were corresponding to the same cell or to neighbouring cells whose fluorescence is bleeding into the neighbour's filter. Such traces were excluded from further analysis (example in figure 3.53).

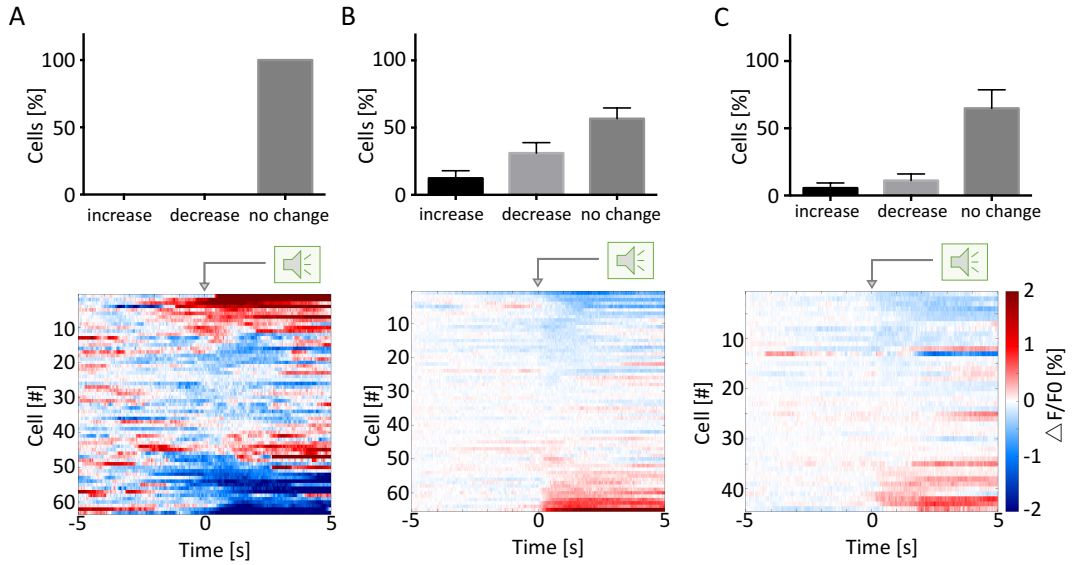

Figure 3.54: CS-responsiveness of PKCδ+ neurons. Top row: percent neurons responding with an increase, decrease, or no change in the fluorescent Ca^{2+} signal. Bottom: heatmaps of fluorescent traces aligned to CS onset. Colours ranging from blue to red code for intensity of fluorescent changes as displayed in the colour bar on the right. Each row represents one cell. CS responsiveness is shown in: ***A*** for habituation, ***B*** first day, and ***C*** second day of two-way active avoidance training.

After having performed all necessary selections, we were left with 63 cells from the habituation session, 65 cells from the first 2wAA training day, and 44 cells from the second 2wAA training day, all coming from a total of six animals. The third and fourth days of 2wAA were not analysed. The fluorescent traces were further aligned to CS-onset, or shuttling onset and changes in fluorescence relative to the corresponding pre-event (10sec) baseline was calculated for each trial separately. We assessed whether the fluctuations in this ΔF/F0 value occurring at the onset of a chosen event type are on average significant by applying a Wilcoxon signed-rank test to the data (see Methods section for further details).

First we aligned the data to the onset of the tone (figure 3.54). During the habituation

session, none of the recorded neurons was to found significantly change fluorescence levels to the tone onset. On the first and second day of conditioning, we found a few responsive neurons, albeit the absolute changes in fluorescence were found to be rather low.

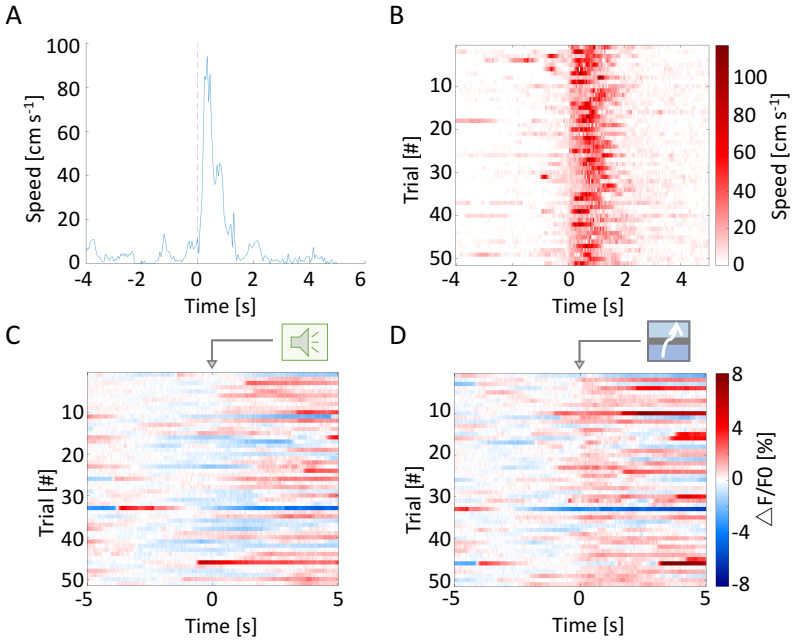

Figure 3.55: Traces aligned to the onset of shuttling. ***A*** Onset of shuttling was determined from the speed profile and is marked with a vertical dashed line. ***B*** Speed profile aligned to onset of shuttling for each of the 51 trials during 2wAA training for one mouse ***C*** An example cell with fluorescent traces aligned to the CS-onset. ***D*** Traces from the same cell, but aligned to the shuttling-onset. Colours ranging from blue to red code for intensity of fluorescent changes as displayed in the colour bar.

We noticed that on the second conditioning day some of the neurons seemed to respond with a variable delay to the tone onset - an example neuron is shown in figure 3.55 C. As the shuttling also occurs at variable intervals after the tone onset, we aligned all traces to the onset of the shuttling motion and saw indeed a better alignment of the responses of the neuron from B to the onset of the shuttling event (figure 3.55 C).

Next, we aligned all recorded neurons to the onset of the shuttling and found responsive neurons on both the first and the second day of 2wAA conditioning (figure 3.56 and figure 3.57). For the first day, we divided the 51 trials into AR and ER+ and looked at neuronal responses during avoidance and US-escape separately. However, the number of AR on the first training day is very low and the average fluorescence changes too noisy. However, when we averaged neuronal responses aligned to shuttles occurring during any of the 51 trials, we found that on average 16.7% of neurons show increased fluorescence,

whereas 45.7% show a decrease (3.56).

On day 2, the ratios were almost exactly reversed: on average 47.0% of neurons had elevated fluorescence signals, whereas 20.5% showed a decrease. Due to small N (6 animals), and high variability, the differences among these mean values were not significant.

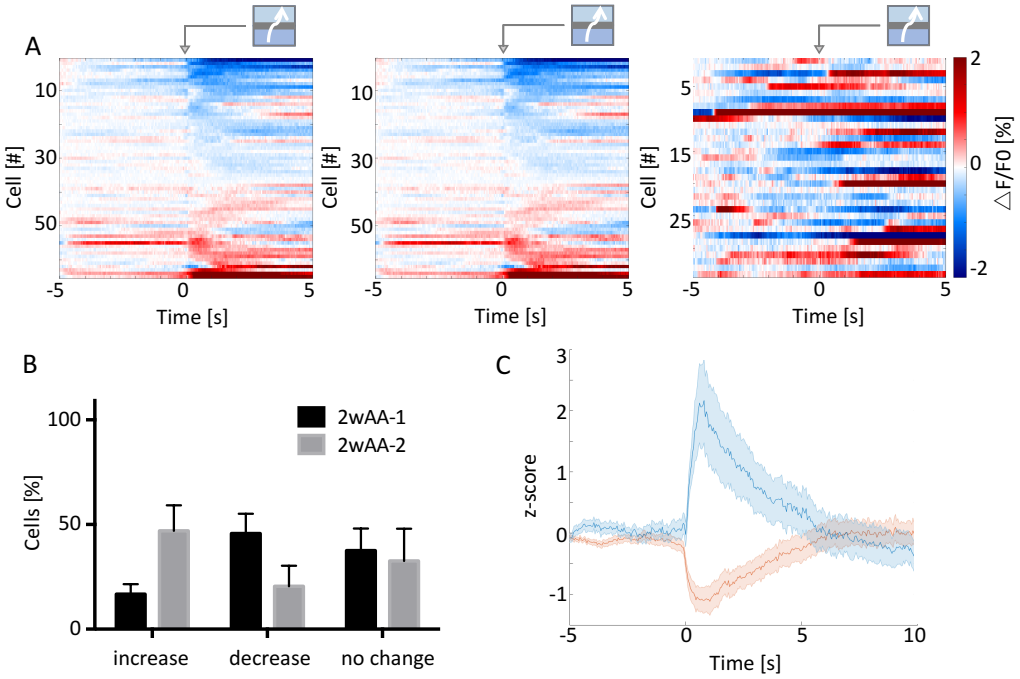

Figure 3.56: Average changes in fluorescence signal, aligned to the onset of the shuttling movement occuring during ***A*** left- any trial, middle- ER+ trials, right- AR trials on the first day of conditioning. Colours ranging from blue to red code for intensity of fluorescent changes as displayed in the colour bar. ***B*** Percent of neurons responding with an increase, decrease, or no change in the fluorescent Ca^{2+} signal to the onset of the shuttling motion (average across all trials) on the first and second day of conditioning. ***C*** Average trace and SEM (shaded) of neurons responding with a significant increase (blue) and decrease (red) in fluorescence to the onset of shuttling on any of the 51 trials on day 1. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test

On the second conditioning day, we again separated the 51 trials into AR type and ER+ type trials. This time, we had sufficient numbers for both trial types. We then compared the percentages of neurons that are responsive to the shuttling onset. Although there was a trend towards a higher fraction of responsive neurons on AR trials than on ER+ trials, we found no significant differences.We then plotted averaged and normalised Ca^{2+} traces (z-scored) from neurons that increase or decrease fluorescent signals upon shuttling on AR trials (figure 3.57). Interestingly, the neurons responded with small,

but prolonged changes in fluorescence.

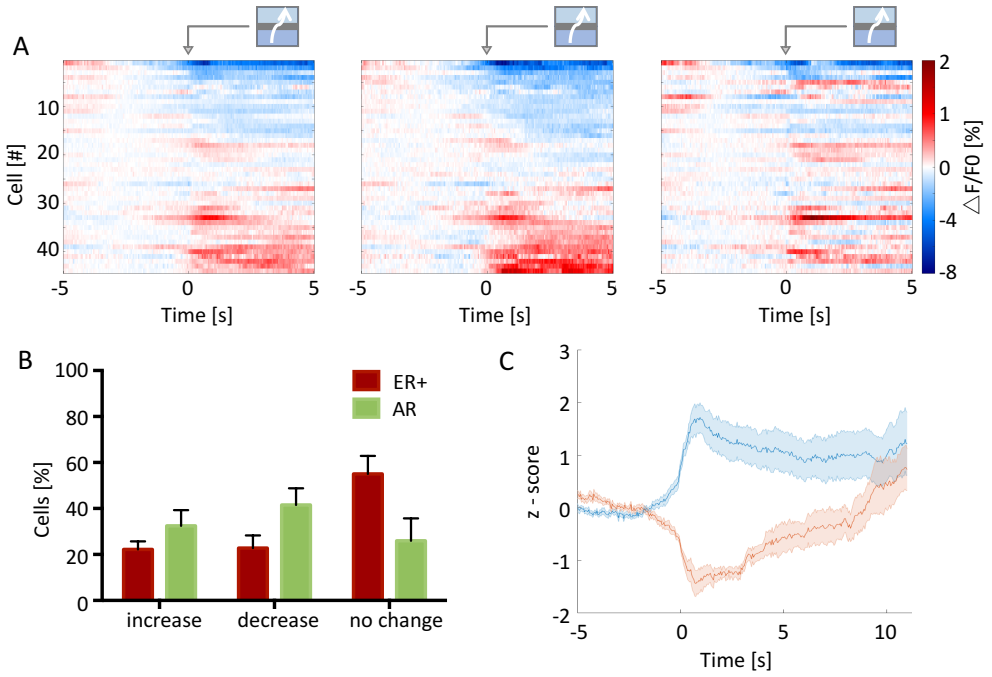

Figure 3.57: Average changes in fluorescence signal, aligned to the onset of the shuttling movement occuring during first day of 2wAA training on ***A*** left- any trial, middle- ER+ trials, right- AR trials. Colours ranging from blue to red code for intensity of fluorescent changes as displayed in the colour bar. ***B*** Percent of neurons responding with an increase, decrease, or no change in the fluorescent Ca^{2+} signal to the onset of the shuttling motion on the first and second day of conditioning. ***C*** Average trace and SEM (shaded) of neurons responding with a significant increase (blue) and decrease (red) in fluorescence to the onset of shuttling on AR trials on day 2. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test.

We were wondering whether the neurons are responsive also to shuttles occurring during the inter-trial interval. We aligned traces to each of the ITI-shuttles and ran significance tests as described, but found no noteworthy responsiveness neither during habituation, nor on the first and second conditioning days (figure 3.58).

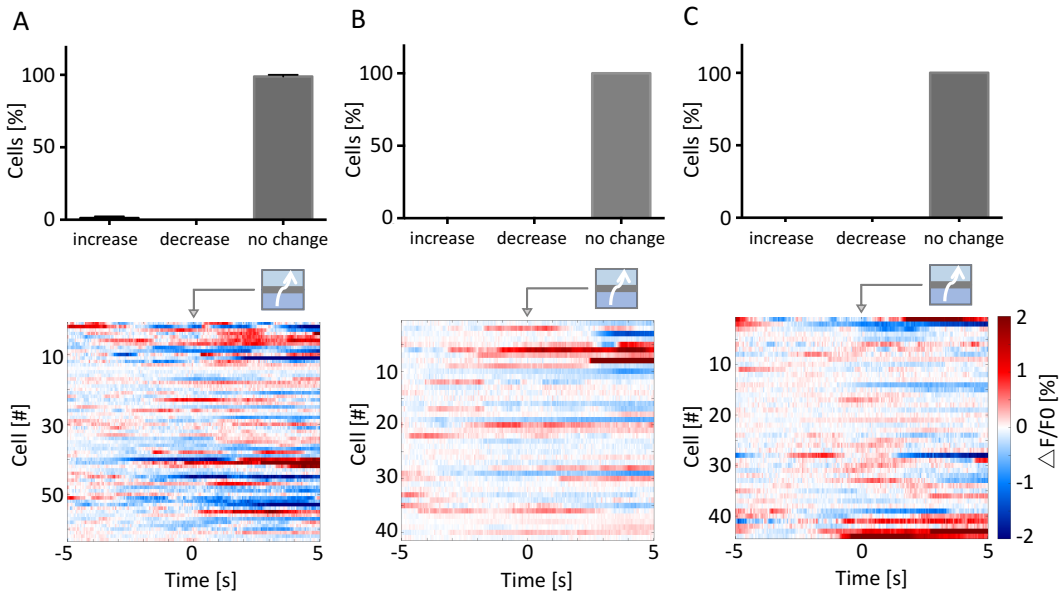

Figure 3.58: Traces aligned to the onset of the shuttling movements that occur during inter-trial-intervals. Top row: percentage of neurons responding with an increase, decrease, or no change in the fluorescent Ca^{2+} signal. Bottom: heatmaps of fluorescent traces aligned to the onset of ITI-shuttling. Colours ranging from blue to red code for intensity of fluorescent changes as displayed in the colour bar on the right. Each row represents one cell. ITI-shuttling responsiveness is shown for: ***A*** habituation session, ***B*** first day, and ***C*** second day of two-way active avoidance training.

Discussion

In this study, a two-way active avoidance paradigm was used with the aim of exploring how different populations of central amygdala (CEA) neurons are involved in expression and learning of active defensive behaviours. Focus was on PKCδ$^+$ and SOM$^+$ neurons. In order to evaluate their contribution to the conditioned defensive system, we manipulated their activity in a spatially and temporally restricted and relatively well-defined manner. We found complex phenotypes that reveal that both types of neurons feed into circuits that control both learning and expression of both active and passive defensive behaviours. A summary of the most important findings is depicted in figure 4.1.

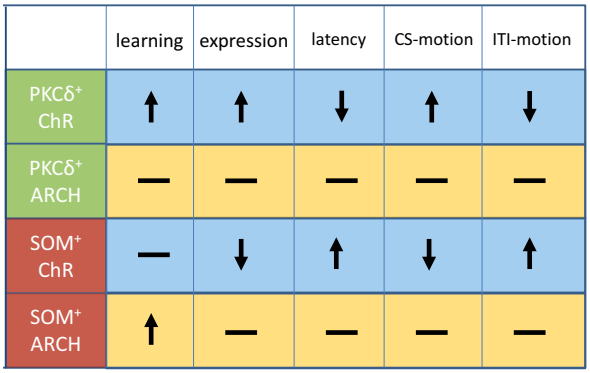

Figure 4.1: Summary of findings from optogenetic studies.

First of all, we have observed an effect of optogenetic activation of ChR expressing PKCδ$^+$ neurons with blue light on unconditioned behaviour. Although freezing was not directly affected during blue light application, the animals did alter their behaviour. This effect needs to be quantified and analysed in detail. Briefly, although blue light does not induce immediate freezing, it does induce an effect on animal's locomotion. Instead of being ignorant to the light based stimulation of PKCδ$^+$ neurons, the exper-

imental subjects come to a halt, slightly extend their extremities and the head, and then start making small, seemingly uncoordinated movements. This looks like several different motor programs are induced at the same time and the mouse switches rapidly among them, unable to perform any of them to completion. The effect of optogenetic activation of PKCδ^+ neurons on behaviour after the light pulse is even more striking. After the light stimulus, robust rebound freezing is induced. Theoretically, there are several possible explanations for this phenomenon. One could for example imagine that any significant decrease in firing of PKCδ^+ neurons is interpreted as a signal to start freezing by the downstream effector neurons and circuits. This would not be in disagreement with their functional identity as CEl_{off} neurons [Haubensak et al., 2010]. It would also not necessarily contradict the lack of effect on behaviour observed upon optogenetic inactivation of these neurons, as CEl_{off} neurons have extremely low baseline firing during habituation. [Ciocchi et al., 2010; Haubensak et al., 2010]
Another option is that the activity of PKCδ^+ neurons is in fact decreased below its original pre-light stimulation baseline after the light pulse, and that this decrease is what controls freezing after the light pulse. Some evidence for this comes from study by Botta et al. [2015] in which prolonged activation of PKCδ^+ leads to a small, but long lasting decrease in firing after the blue light stimulus (also see figure 3.3). On molecular level this could be achieved by a flooding of the system with GABA after a prolonged activation of the inhibitory PKCδ^+ neurons. A high increase in extracellular GABA would have an effect on exrasynaptic GABA receptors that can have slow kinetics. Additionally, removal of high concentrations of extracellular GABA would take some time, meaning that post-light inhibition of PKCδ^+ neurons would last for a while, thus allowing a prolonged freezing after the light offset.

The most striking effects that we achieved with optogenetic activation of PKCδ^+ neurons are however the boost of both 2wAA learning and expression. Furthermore, optogenetic activation decreases the latency of responding to the CS. Related to this is an effect on locomotion during the CS: we observe an increased average speed of movement during the CS. As we concurrently observe shorter avoidance/escape latencies, we cannot make conclusions about whether this means that the animals increase motion during the entire duration of the CS, or whether the observed effect can be explained purely by an earlier onset of escape/avoidance and the resulting increase in contribution of the shuttling movement to the average CS-speed. In order to be able to differentiate between the two possibilities, we would need to have a more detailed look at the speed profile during the

CS. As the CSs have different durations, we would first need to categorise them into AR and ER+ trials. Then for each trial type, we would need to separate the speed profile into at least two parts. In the first part of the CS, we would include CS-speed before the onset of the shuttling movement. In the second part of the CS, we would include only the CS-speed after and including the onset of the shuttling movement. It is an interesting and important analysis that we will perform in the future.
The inter-trial-interval motion was also affected by ChR-based activation of PKCδ$^+$ neurons. As the ITI-speed was not significantly altered, we used freezing as a more specific measure of animal motion. Here we saw a peculiar increase in ITI-freezing in the manipulated cohort, which was independent of whether the manipulation occurred during the directly preceding trial or not. It is disputable why the ITI-freezing is higher in the manipulated cohort.

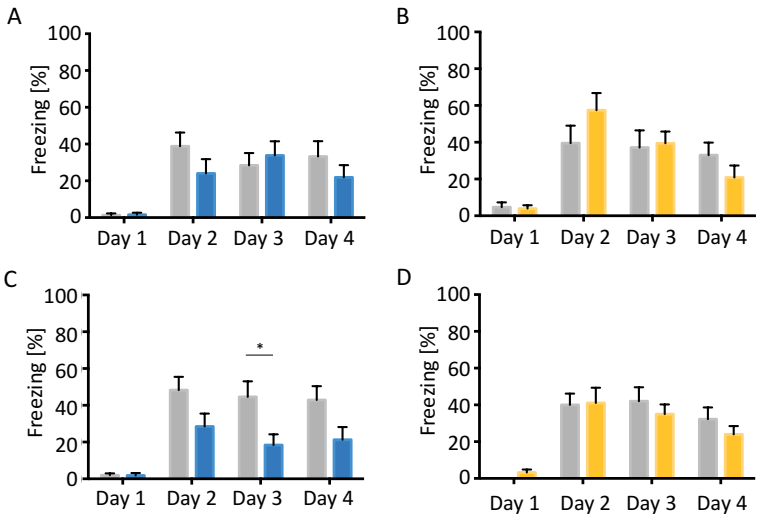

Figure 4.2: Contextual freezing before the onset of the first CS on all four conditioning days in ***A*** the optogenetic activation of PKCδ$^+$ neurons. ***B*** the optogenetic inhibition of PKCδ$^+$ neurons. ***C*** the optogenetic activation of SOM$^+$ neurons. ***D*** the optogenetic inhibition of SOM$^+$ neurons. ChR group is shown in blue, ARCH group in yellow and EGFP groups are in grey. Statistical tests: repeated measures two-way ANOVA with post-hoc Sidak test.

For example, it could be that not just the ITI freezing, but also the contextual freezing was altered. This could be a hint to CS-idependance of ITI freezing and point to long-term changes in general arousal and perceived threat-levels that are altered by the blue light manipulation. However, analysis of contextual freezing as shown in figure 4.2 A, did not confirm this hypothesis. Although the differences were not significant, there

was a tendency towards lower freezing in the ChR-group on day 2. An effect that is anti-correlated with the observed increase in AR rates and with the oserved increase in ITI freezing.

Another idea worth exploring is to examine the correlational structure of ITI freezing with the avoidance response rate in the unmanipulated cohort. It is conceivable that higher ITI freezing is correlated with higher AR rates. Even if true, this would not convincingly clarify which of the two is cause and which the effect. If one were to analyse a hypothetical difference in freezing during the very first ITI on trials when animals performed an AR or ER+, one would be coming closer to a statement about cause and effect.

Intriguingly, optogenetic inhibition of CEA PKCδ^+ neurons did not lead to any observable effect on neither learning, nor expression of active avoidance. The latency to shuttle during CS was not altered, and neither was the speed of motion during the CS, nor the measured motion parameter during the CS. Furthermore, contextual freezing was not altered (figure 4.2), nor were there any observable effects of light on naive animals during habituation. This is all true although we know that using the same viruses, fibres and light application parameters results in reliable inhibition of PKCδ^+ neurons, as published in Botta et al. [2015]. Interestingly, inhibition of CEA PKCδ^+ neurons is known to have the potential to influence anxiety and feeding in mice.[Botta et al., 2015; Cai et al., 2014]. Thus these neurons do have a proven potential to influence animal behaviour in bi-directional manner. It is not clear why this is not the case for active avoidance. In fact, it might be that the lack of effect is not specific to active avoidance only, but to active escape, as Fadok et al have also not observed effects of PKCδ^+ neuron inhibition on performance in the conditioned flight paradigm. This could on one hand be explained simply with a statement that PKCδ^+ neurons are naturally not involved in escape/avoidance behaviours. In this case the effects observed in the ChR group could be interpreted as an artefact of exaggerated, unnatural highjacking of the active defence pathway. Another explanation is that they are not necessary for avoidance in this particular setting, but that the strong effect of ChR activation is observed because the PKCδ^+ neurons have a natural access to the avoidance pathways that is exploited in other circumstances. The lack of effect of ARCH mediated inhibition in this scenario could be explained for example with the argument that if PKCδ^+ are naturally not changing their firing to the CS in these particular circumstances, there is simply no effect for further decreasing their firing with ARCH. Another option is that the effector regions and neurons that PKCδ^+ communicate with are preferentially tuned to reacting to strong increases in inhibition from (and not inhibition of) the PKCδ^+ neurons. We

observe such strong increase in inhibitory drive from PKCδ$^+$ neurons during ChR based manipulations of this population. It could be that a decrease in firing of ca. 2Hz, as is observed after PKCδ$^+$ inhibition in naive animals, is below the threshold needed to cause an effect in the downstream areas responsible for active defence. It could be for example, that if one were to inhibit firing of PKCδ$^+$ neurons during a state in which they fire with a higher tonic activity level, the effect on the downstream targets could be bigger. If one were to assume that tonic firing of PKCδ$^+$ neurons is greater on more advanced conditioning days, but before the behaviour becomes habitual, one could imagine that inhibiting firing of PKCδ$^+$ neurons would have a greater impact on the total amount of received inhibition of downstream target neurons.

A different line of argumentation is that there is a possibility that our particular light application parameters are not ideally suited to influence aspects of avoidance behaviour that PKCδ$^+$ neurons have the potential to alter. The light application pattern is in fact a deliberately chosen compromise between a theoretically most effective stimulation for influencing learning, and a theoretically most effective stimulation for catching effects on expression of active avoidance. Manipulations of neural activity on every trial of day 1 would be better suited to catch influences on learning, by assessing the AR rate on the second conditioning day. On the other hand, considering the 2wAA learning curve, it would be probably better to leave out manipulations on day one, but instead apply them only on the second conditioning day.

The optogenetic manipulations of SOM$^+$ neurons largely mirror the effects of the same manipulations on PKCδ$^+$ neurons. This is in line with a mutually inhibiting functional and anatomical network that these neurons are known to be a part of. [Ciocchi et al., 2010; Fadok et al., 2017; Haubensak et al., 2010] The observed asymetry in behavioural consequences of optogenetic activation and inhibition of SOM$^+$ neurons is puzzling. One could employ some of the same arguments named above for the lack of effect during optogenetic inhibition of PKCδ$^+$ neurons. Briefly, most likely this is either due to the optogenetic manipulation parameters used in the study, or due to the peculiarities of downstream effectors of SOM$^+$ neurons from within or from outside the central amygdala.

The Ca^{2+} imaging of PKCδ$^+$ neurons during 2wAA yielded results that are somewhat challenging to interpret. We observe significant changes in the fluorescent signals, mostly upon shuttling. However, the observed changes have very small amplitudes and are only obvious after averaging across many trials. As most PKCδ$^+$ neurons are CEl_{off} neurons, we have to take into consideration that their tonic firing has previously observed to be around 6Hz in certain situations [Ciocchi et al., 2010]. Combined with the slow

dynamic of the GCaMP6s sensors [Chen et al., 2013], this might mean that temporary changes in firing resulting in rate changes of only several Hz might be barely detectable with GCaMP6s. Faster sensors like GCaMP6f are less bright, but might provide better temporal resolution necessary to allow more confident detection of changes in neuronal firing. Additionally, the unknown Ca^{2+} dynamics inside of PKCδ^{+} neurons might be unfavourable in combination with the GCaMP6f sensor. The still relatively low N gathered during Ca^{2+} imaging experiments are another hurdle to making confident conclusions from our studies. Although we do not observe significant changes in ratios of responsive cells from day one to day two, there is a strong trend towards more neurons that increase fluorescent signals during trials of day 2 in relation to numbers observed on day 1. This increase might be what is driving successful performance of the two-way active avoidance task. Additionally, if this difference were to turn out to be true, it would be in line with the observed boosting effects of optogenetic activation of PKCδ^{+} neurons on expression and learning of 2wAA. Interestingly, we also do not observe significant differences in fractions of responsive neurons between AR and ER+ trials. There is however a strong trend towards more responsive neurons during AR trials. There is still a lot of analysis that can be done with the data. For example, we currently do not follow the identity of neurons from habituation to the first and second conditioning day. Although we do not observe significant differences in total numbers of responsive neurons from day 1 to day 2, it could still be that shifts revealed by classifying neurons based on their functional identity could occur. Such shifts could only be observed by analysing the neurons' full response profiles over the entire conditioning procedure.

In conclusion, using optogenetics and imaging, we confirm that central amygdala mediates active defensive behaviours. Furthermore, we specifically identify that PKCδ^{+} neurons not only regulate passive, but also active defensive behaviours. PKCδ^{+} neurons are thus in a unique position of power, which allows them to influence very different defensive strategies acutely and flexibly. Additionally, they also optimise adaptation to threatening situations in the long run via their lasting effects on learning.

Methods

5.1 Animals

Experimental animals were 2-5 month old transgenic mice back crossed to C57BL/6j line (Charles River Laboratories) for at least five generations. SSTtm2.1(cre)Zjh (SOM-ires-Cre) transgenic line was originally obtained from Jackson Laboratories, PKC-δ::GluClα-iCre (PKC-δ-ires-Cre) [Haubensak et al., 2010] mice were obtained from the lab of David J. Anderson (Caltech). All animals used in behavioural experiments were individually housed after the surgery in a 12 h light / 12h dark cycle. Food and water were available ad libitum. All behavioural procedures were performed during the light cycle. Experimental subjects were treated with care and respect. All animal procedures were performed in accordance with institutional guidelines and were approved by the Veterinary Department of the Canton of Basel-Stadt, Switzerland.

5.2 Viral injections and implantations

Anaesthesia was induced with 4% and maintained with 1.5% isoflurane (Attane, Provet AG, Switzerland) in oxygen-enriched air (Oxymat 3, Weinmann Geräte für Medizin GmbH + Co. KG, Germany). Before starting the surgical procedure, sufficient analgesia was insured with intraperitoneal injections of meloxicam (100µl of 0.5 mg/ml Metacam, Boehringer Ingelheim Pharma GmBh + Co. KG, Germany) and subcutaneous injections of ropivacain (100µl of 2mg/ml Naropin, AstraZeneca, UK) under the scalp. Core body temperature was maintained at 36.5°C with a feedback-controlled heating pad (FHC). Animals were fixed in a stereotactic frame (Model 1900 Stereotaxic Alignment System, David Kopf Instruments, Tujunga, CA, USA), and 0.3mm diameter precision holes were drilled into the skull above the area of interest. Care was taken not to perforate the dura at this point. Stereotaxic coordinates used for CEA injections: 1.20mm posterior to

bregma, 0.95mm lateral to the midline suture and -4.10 to -4.05mm below the cortical surface. Virus was filled into calibrated glass pipets (5µl microcapillary tube, Sigma-Aldrich Inc., St.Louis, MO, USA) and 30-100nl were injected using a pressure-injection system (Picospritzer III, Parker, Hollis, NH).
For optogenetic manipulations during behavior, mice were bilaterally implanted with optic fibre connectors (fibre: 0.48 numerical aperture, 200 mm diameter, Thorlabs, Newton, NJ, USA) a week after the virus injection. Dura was removed with a sterile needle and fibres were lowered to 500-300m above the injection sites and the implants were fixed to the skull with micro-screws, cyanoacrylate glue (UltraGel, Henkel, Düsseldorf, Germany) and blackened dental cement (Paladur, Heraeus GmbH, Hannau, Germany). Animals were granted at least two-weeks of recovery after the implantation. In order to achieve optogenetic manipulations during behaviour, the implanted fibre connectors were connected to a custom-built laser bench (MBL473, 473 nm wavelength laser MBL573, and a 593.5nm wavelength laser MGL593.5, both from CNI Lasers). The laser power at the tip of the fibre stub was adjusted to match 10-15mW.
For Ca^{2+} imaging during behavior, mice were unilaterally implanted with a GRIN lens (0.6mm diameter, 7.3mm length, Inscopix Inc., Palo Alto, CA, USA) a week after the virus injection. Similarly to fibre connectors, they were carefully lowered to the injection site using a micropositioner and then fixed to the skull with micro-screws, glue and blackened dental cement (Paladur, Heraeus Gmbh, Hannau, Germany). The lens surface was protected using 2-component casting silicone until the imaging. The animals were allowed to recover for two weeks, after which the expression levels were checked with a microendoscope on a weekly basis up until fluorescence levels were sufficient for imaging. At that point, baseplate (Inscopix Inc, Palo Alto, CA, USA) was mounted using flowable dental composite (Vertise Flow, Kerr) under light (1.5%) and brief (ca.15min) isoflurane anaesthesia. After 24h of recovery, the animals were handled daily in order to accustom them to the brief head fixation procedure necessary for miniature microscope mounting.

5.3 Viruses

Selective gene expression was achieved by packaging conditional, Cre-depented constructs into recombinant adeno- associated viruses (rAAV) and injecting these into the desired target sites in SOM-ires-Cre and PKCδ-ires-Cre mice.

Expression of excitatory channelrhodopsin-2 was achieved with following viruses:
- rAAV2/5.EF1.dflox.ChR2(H134R)-eYFP.WPRE (V1550, PennVector Core)
- rAAV2/5.EF1.DIO.ChR2(H134R)-mCherry.WPRE (V1449, PennVector Core)

Inhibitory opsin expression was accomplished using:
- rAAV2/9.CBA.FLEX.ARCH-GFP (V1615, PennVector Core)

Expression of fluorescent marker genes was achieved with:
- rAAV2/1.CAG.FLEX.EGFP.WPRE.bGH (V1675, PennVector Core)
- rAAV2/9.CAG.FLEX.tdTomato.WPRE.bGH (V1682, PennVector Core)

Expression of genetically encoded Ca^{2+} indicators was achieved with:
- rAAV2/9.CAG.FLEX.GCaMP6s

All rAAVs were purchased at the University of Pennsylvania, Penn Vector Core, unless noted otherwise.

5.4 Behaviour

Behaviour apparatus

Auditory signaled two-way active avoidance training was performed in square plexiglass shuttle boxes of 25cm length and 25cm width. The boxes were separated into two equal compartments by placing a gray, plastic divider with a constantly open passage of 6 cm width for optogenetics experiments. The width of the passage was extended for microendoscope imaging experiments to 8cm in order to allow easy passage of animals mounted with portable scopes. Two separately controllable metal grid floors were controlled with one animal precision shocker each (H13-15, Coulbourn Instruments, Holliston, MA, USA). A very mild 0.3 mA AC scrambled footshock was used as a US. A loudspeaker was placed in the middle of the shuttlebox and a 5kHz continuous tone was used as a CS. Sound intensity was measured in the corners and at passage site and adjusted to be between 70 and 75 dB. Sounds were generated with System 3 RP2.1 real time

processor, SA1 stereo power amp, and RPvdsEx software (all from Tucker-Davis Technologies, Alachua, FL, USA). The shuttlebox was housed within a sound-attenuating chamber. The chamber was lit by four white light LEDs set to low luminosity levels, attached in the top corners of the sound-attenuating chamber. In order to provide optimal illumination for tracking, VIS LEDs were supplemented with four IR LEDs one coming from each side of the chamber. Additionally, red LED strips were placed just outside and below the grid floor in order to eliminate shadows. Animal motion was monitored using Viewer2 software (Biobserve, Bonn, Germany). Viewer2 was also used to control the application of TTL pulses needed to trigger tone, shock, lasers, start and stop of electrophysiology or Ca^{2+} imaging, using the HomeCage/DualCage plug-in, in a fully automatic, dynamic, behaviour-dependant manner. During Ca^{2+} imaging, behaviour was additionally recorded using a second camera and the Cineplex tracking software.

Two-way active avoidance conditioning

All animals were allowed to get comfortable to being picked up and gently handled by the experimenter during four to five 10 minute long daily handling sessions directly preceding the conditioning. The animals were then given a day to rest before the onset of the training procedure. On the first day, the animals were placed into the shuttlebox and allowed to freely explore the context for 20minutes. This exploration period was followed by five presentations of a 10sec 5kHz continuous 70-75dB tone (future CS). Three minutes later, the animals were taken out of the shuttlebox, which ended the habituation procedure. 24h later, the 2wAA training began, which consisted of five consecutive conditioning days. Each training day consisted of 51 trials, with an inter-trial-interval (ITI) of 45sec. On each trial, the CS was presented which had a maximum duration of 10sec. If the animal shuttled pass the divider during the first 5sec of the CS, the CS was terminated immediately and was not followed by a US. This type of conditioned response was classifed as an avoidance response (AR^{+}). If however the experimental subject did not not shuttle, a US with a maximum duration of 5sec began. If shuttling occurred during the 5sec, the CS and the US were immediately co-terminated. This type of unconditioned response was classified as a successful escape response (ER^{+}). If the animal did not not shuttle at all during a trial, the lack of shuttling was classified as an unsuccesful escape response (ER^{-}). Freezing data was extracted using the Viewer2 Freezing plugin. All behavioural data was analysed using a custom- written MATLAB 2016a (The Mathworks Inc, Natick, MA, USA) analysis software.

Optogenetic activity preturbations during and 2wAA

During the optogenetic manipulation experiments, the training procedure was essentially the same as described above, but with slight changes. After the five CS presentations, blue or yellow light was presented five times for 10sec, before the end of the protocol was initiated. On the training days, every second trial coincided with presentations of blue or yellow light, meaning that the light was presented at exactly the same time as the tone. In some of the animals, five light-only trials were presented at the end of the last conditioning session. All optogenetics experiments were ran with balanced experimental groups that consisted of both ChR2 or ARCH expressing "experimental" animals and the EGFP expressing "control" animals. After the virus injection, the identity of the injected virus was conceiled, such that during behavioural training the experimenter was unaware of weather the animals belonged to experimental or control groups.

5.4.1 Single-unit recordings

Two weeks after injection of AAVs with conditional ChR2 or ARCH construct, optrodes that allow simultaneous extracellular recordings and optogenetic light stimulation were implanted as described above. Optrodes consisted of fiber optic connectors to which 16 insulated, 13µnichrome wires were gold-plated until they reached animpedance of 30-100 kΩ(Sandvik, Stockholm, Sweden) and were then attached to an 18-pin Omnetics Connector (Omnetics Connector Corporation, Minneapolis, MN, USA). After a minimum recovery period of two weeks following the surgery, omnetics connectors were attached to a headstage (Plexon Inc, Dallas, TX, USA) with 16 unity-gain operational amplifiers. The headstage was connected to a preamplifier (Plexon Inc, Dallas, TX, USA) and the signals were aimplified with a 100x gain, and then band-pass filtered from 150Hz to 9kHz. The signals were digitised at 20kHz and band-pass filtered from 250Hz to 8kHz using a Multichannel Acquisition Processor system (Plexon inc, Dallas, TX, USA). Action-potential like signals were then isolated by time-amplitude window discrimination and template matching. Subsequently, brief pulses of blue light (60 x 10 ms and 60 x 300 ms for ChR2) or yellow light (120 x 300 ms for ARCH) were applied in order to identify PKCδ^+ neurons.

Single-unit spike sorting

Spike sorting was performed using the offline sorter software (OFS4, Plexon Inc, Dallas, TX, USA). First the data was cleaned up manually by removing clipped waveforms and AP-atypical signals. Principal components (PC) were calculated for all remaining waveforms and then the first three PCs were plotted in a three dimensional scatter plot. Clusters containing similar valid waveforms were visually identified and manually defined. The quality of selected clusters was assessed using the J3 statistics, which corresponds to the ration of between-cluster to within-cluster scatter. Davies-Bouldin validity index represents the ration of the sum of within-cluster scatter to between-cluster separation. Thus a high J3 and and a lot Davies-Bouldin scores are indicative of good unit isolation. Any units with a refractory period of <1ms were not taken into further analysis. The stability of units over different recording sessions were quantified by calculating pairwise linear correlation (r) between waveform shapes from two different sessions.

Analysis of single unit data

In order to avoid redundant analysis of the same neuron recorded on different channels, cross- correlation histograms were caculated using the NeuroExplorer 5 software (Nex Technologies, Madison, AL, USA). If two neurons were found to be simultaneously active with this analysis, only one of the two neurons was taken into account for further analysis. Neuronal activity of the recorded neurons was approximated by calculating frequency or normalized activity scores (z-score transformation). The baseline used for normalisation was the 300ms pre-stimulus baseline. z- scores were averaged across all trials of the same type. The neurons were regarded as belonging to PKCδ^+ population if they responded to blue light pulses (ChR2 group) with an increase in firing (z-score ≥ 2) within 8 ms after light onset or if they responded to yellow light pulses (ARCH group) with a decrease in firing (z-score < -1) within 10 ms after light onset.

5.5 Immunohistochemistry

The mice were deeply anaesthetised with Avertin and then perfused with 15ml ice-cold PBS, followed by perfusion with 50ml ice-cold 4%PFA in PBS. Brains were extracted from the skull and post-fixed for either 4h or o/n at 4°C. A Leica precision vibratome

(Leica Microsystems, Heerbrugg, Switzerland) was used to cut 80um thin brain sections. Sections from brains of experimental animals that were included in the optogenetics, single unit and microendoscope imaging experiments were stained with DAPI, rinsed three times for 15min in PBS at room temperature and mounted onto gelatine-coated glass slides using Fluostab embedding medium.
Sections used for histological cell-identity analysis were rinsed with 0.5% PBS-T (0.5% Triton-X in PBS) three times for 15min and subsequently incubated in a blocking solution (20% normal horse serum in 0.5% PBS-T) at room temperature. After blocking, the sections were placed in primary antibody solution, consisting of a primary antibody diluted in 0.5% Triton-X in PBS with 1.5% normal horse serum. After a 48h incubation period at 4°C, the slices were rinsed three times with a 0.5% PBS-T solution and subsequently placed in secondary antibody solution consisting of the secondary antibody diluted in 0.5% Triton-X in PBS with 1.5% normal horse serum. After a 24h incubation at 4°C, the slices were stained with DAPI and rinsed three times for 15min in PBS at room temperature. All slices were mounted onto gelatine-coated slides using Fluostab embedding medium.

goat anti-GFP	at 1:500 (Abcam, Cambridge, UK)
rabbit anti-GFP	at 1:500 (A11122, Life Technologies, Carlsbad, CA, USA)
mouse anti-PKCδ	at 1:500 (610398, BD Biosciences, NJ, USA)
mouse anti-SOM	at 1:500 (MAB354, Merck Millipore, Billerica, MA, USA)

All secondary antibodies were used at 1:500 dilution and were obtained from Thermo Fisher Scientific, Waltham, MA, USA

donkey anti-goat IgG - Alexa Fluor 488 (A11055);
donkey anti-rabbit IgG - Alexa Fluor 594 (A21207)
donkey anti-mouse IgG - Alexa Fluor 568 (A10037)
donkey anti-mouse IgG - Alexa Fluor 647 (A-31571)

5.6 Fluorescent microscopy

In order to confirm the extent of virus expression and fiber optic, electrode and GRIN lens placement, sections stained only with DAPI were imaged using an epifluorescent automated microscope Axio Scan Z1 (Carl Zeiss AG, Oberkochen, Germany) with an objective with 5x magnification and 2x2 binning using transmission light, as well as filter sets recommended for DAPI, GFP and/or when applicable RFP imaging. Additionally some slices were imaged with a LSM700 confocal microscope (Carl Zeiss AG, Oberkochen, Germany) using 10x and 40x oil-immersion objectives.

5.7 Analysis of Ca^{2+} imaging data

Each frame acquired with the microendoscope nVista (Inscopix Inc, Palo Alto, CA, USA) was filtered using an ImageJ Fast-Fourier-Transform bandwidth filter. Motion correction was performed using the Mosaic v1.1.3 software (Inscopix Inc, Palo Alto, CA, USA). Extraction of fluorescent traces was performed using automatically detected individual cell filters, based on combined principal and individual component analysis, as implemented in the Mosaic software and as described in Mukamel et al. [2009]. The cell masks and extracted cell traces were exported to MATLAB 2016a (The Mathworks Inc, Natick, MA, USA) and further processed there with custom-written analysis scripts. First, all duplicate, overlapping image filters or filters corresponding to a cluster of cells rather than a single cell were removed from the analysis. Second, all traces containing residual motion artefacts were removed. Third, all traces containing potential bleed-through from a neighbouring cell were removed from further analysis. Each trace was then de-trended by subtracting the eighth percentile value of the fluorescence distribution in a 60sec sliding window from the raw fluorescence signal. $\Delta F/F0$ was calculated as (F-F0)/F0, where F0 is the median of the 10sec directly preceding an event of interest. To obtain the change in fluorescence in percent, the $\Delta F/F0$ was multiplied by 100. In order to calculate z-scores of Ca^{2+} traces for each event, the baseline for each trial was defined as 10sec directly preceding an event of interest, then the standard z-score transformation was performed. For that, the fluorescent value at each time point, minus the mean of the baseline fluorescence were divided by the standard deviation of baseline fluorescence values. In order to test if a cell responded significantly to all an event of a certain type, Wilcoxon signed-rank test was used to compare the last second of the baseline with the first second after the onset of the event.

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